Sara Daniela Azevedo Costa

Diversidade da comunidade bacteriana da pele da rã verde

Diversity of the cutaneous bacterial community in the Perez' frog

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Sara Daniela Azevedo Costa

Universidade de Aveiro
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Departamento de Biologia

Sara Daniela Azevedo Costa

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Diversity of the cutaneous bacterial community in the Perez' frog

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar e Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Paula Maria Vasconcelos Morais, Professora Auxiliar do Departamento de Ciências da Vida da Universidade de Coimbra.

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Presidente

Prof. Doutor Carlos Fonseca
Professor Associado do Departamento de Biologia de Universidade de Aveiro

Prof. Doutor António Manuel Veríssimo Pires
Professor Auxiliar do Departamento de Ciências da Vida da Universidade de Coimbra

Doutora Isabel Maria Cunha Antunes Lopes
(Orientadora)
Investigadora auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro

Prof. Doutora Paula Maria Vasconcelos Morais, (Co-Orientadora)
Professora Auxiliar do Departamento de Ciências da Vida da Universidade de Coimbra

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palavras-chave Amphibia; *Pelophylax perezi*; Microbioma cutâneo; Variação intra-populacional; Contaminação por metais

resumo A pele de alguns anfíbios possui uma comunidade bacteriana residente que pode conferir tolerância a stress ambiental. A contaminação química pode causar efeitos adversos diretos em anfíbios, mas ao mesmo tempo pode reduzir a diversidade bacteriana da pele e, consequentemente, alterar a sensibilidade destes organismos a futuras perturbações ambientais. Compreender a estrutura, dinâmica e especificidade desta comunidade microbiana é necessário para desenvolver uma melhor e mais ampla proteção deste grupo de organismos. Assim, o presente estudo teve como objetivo geral investigar a comunidade bacteriana associada à pele da rã verde comum *Pelophylax perezi* (Seoane) através da análise da variação intra- e inter populacional. Para alcançar este objetivo o microbioma exterior das rãs foi caracterizado pelo método independente de cultura (PCR / DGGE) e avaliada também a parte cultivável de bactérias. Além disso, para avaliar os efeitos causados pela exposição a contaminação química na comunidade bacteriana da pele, 30 isolados bacterianos foram expostos a um efluente ácido e rico em metais. As zaragatoas para amostrar as bactérias simbióticas da pele dos anfíbios foram recolhidas em 28 indivíduos de cinco locais diferentes, um deles impactado por um efluente ácido e rico em metais (Ribeira da Água Forte, Aljustrel). Para cada local de amostragem foi realizada a caracterização físico-química de amostras de água. O método independente de cultura mostrou um perfil característico de rãs do local contaminado e que a variabilidade tanto intra- e inter- populacional existe no microbioma da pele da rã *P. perezi*, no entanto, a última parece ser maior do que o anterior. Avaliando a porção cultivável de bactérias, a concentração microbiana por anfíbio varia entre animais do mesmo meio e entre os animais de diferentes ambientes. Os resultados revelaram uma baixa diversidade e densidade de bactérias (UFC / zaragatoa) em indivíduos que habitam o local contaminado por metais. As bactérias isoladas foram geneticamente identificadas com base na sequência do gene de rRNA 16S. O ensaio ecotoxicológico de exposição de isolados bacterianos ao efluente ácido e rico em metais mostrou que a percentagem de isolados resistentes foi maior em isolados provenientes de animais da área impactada pelo efluente. Foi ainda observado que isolados bacterianos quando expostos ao efluente têm um atraso na sua taxa de crescimento comparativamente ao controlo.

keywords Amphibia; *Pelophylax perezi*; skin microbiome; within populations variation; metal contamination

abstract Amphibian skin holds a resident bacterial community in the skin surface may confer amphibian species tolerance to environmental stressors. Exposure to chemical contamination may cause direct effects to the amphibians but, simultaneously, may reduce skin bacterial diversity and consequently alter the sensitivity of amphibians to future environmental stressors. Understanding the structure, dynamics and specificity of this microbial community is needed to engage a better and broader protection of amphibians. Accordingly, the present study aimed at investigating the skin-associated bacterial community of the Perez´s frog *Pelophylax perezi* (Seoane) looking at among and within population variation. To attain this main goal the outer microbiome of the frogs were characterize by culture independent method (PCR/DGGE) and assessing the cultivable portion of bacteria. Furthermore, to evaluate the effects caused by exposure to chemical contamination in the skin bacterial community, some bacterial isolates were exposed to a rich metal contaminated effluent. Skin swabs for sampling symbiotic skin bacteria were collected from 28 amphibian individuals from five different ponds, one of them a metal-rich contaminated effluent (Ribeira da Agua Forte, Aljustrel). For each sampling site physical and chemical characterization of water samples was carried out. A culture independent method showed a characteristic profile in frogs from contaminated site and that both intra- and inter-population variability exist in amphibian skin microbiome. Assessing the cultivable portion of bacteria, microbial concentration per amphibian varied within animals from the same environment and between animals from different environments. Results revealed low diversity and bacterial density (CFU/ frog swab sample) on individuals from metal contaminated site. Isolated bacteria were genetically identified based on 16S rRNA gene sequence. Ecotoxicological assays exposing 30 bacterial isolates to the metal contaminated effluent showed that the percentage of resistant isolates was higher in frogs from the contaminated site. It was also observed that those bacteria exposed to effluent presented a delay in their growth rate.

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1. Introduction

Amphibian decline overview

The Global decline of amphibians is of high concern in the scientific community regarding the conservation of threatened species and loss of biodiversity within this taxonomic group (Stuart *et al.* 2004; Wake & Vredenburg 2008). As reviewed by a few authors (e.g. Blaustein & Kiesecker 2002 and Beebee & Griffiths 2005) several factors have been pointed as causative of this worldwide decline. Among the major threats the following were identified: the habitat destruction and fragmentation, disease, climate change, environmental pollution, direct exploitation for the food, medicine and pet trades, increase in UV-B irradiation due to anthropogenic ozone depletion, and invasive species. However, recent studies directed at this problem, suggest that global amphibian losses, at present, is mainly due to increased disease susceptibility caused by the actual environmental scenarios (Pounds *et al.* 2006; Fisher 2007).

Amphibians are capable of inhabiting a wide range of different habitats, ecosystems, and climatic regions (e.g.: deserts, mountains, temperate or tropical regions), which suggests the wide variety of environmental adaptations, diversity, and functions in distinct ecosystems. Some species are distributed across a wide geographic area and live in a variety of habitats, being almost ecological generalists (Loureiro *et al.* 2008; IUCN 2008).

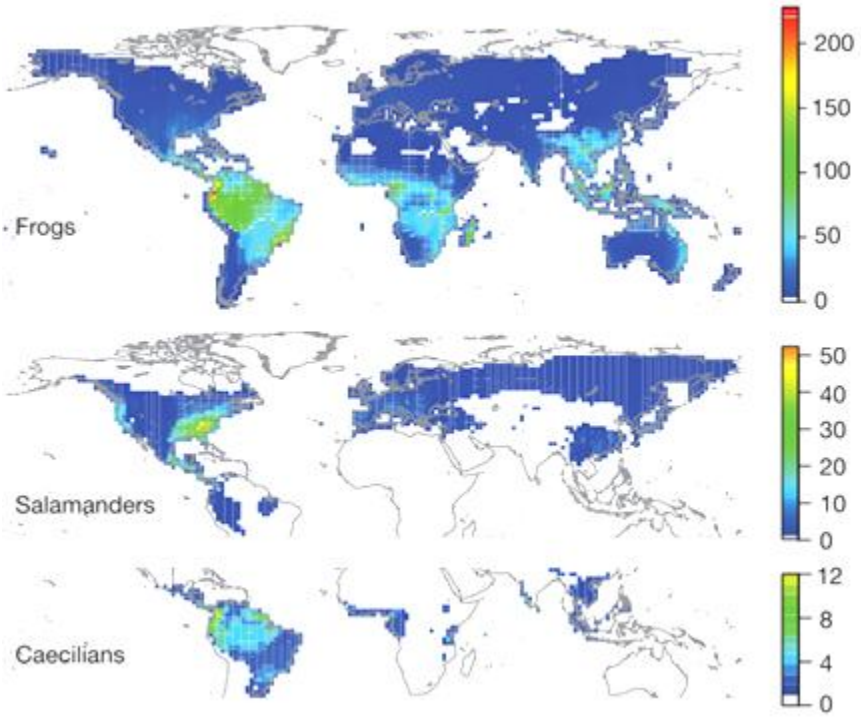

Figure 1 - Current spatial variation of species richness (number of species per grid cell) of Anura (n= 4,875), Urodela (n= 508) and Gymnophiona (n=144). (Illustration from Hof *et al.* 2011)

Others have much more restricted distribution ranges. Otherwise, some species have more specialized habitat requirements, which mean that they are particularly susceptible to environmental alterations, especially if suitable habitats are scarce, patchy in their distribution or limited geographically (Wijesinghe & Brooke 2004).

Distribution, diversity and abundance are not equal or linear, and seem to have variants with latitude and altitude. Accordingly with information available in some published reports and clearly demonstrated in Figure 1, there are trends for the amphibian distribution and significant biogeographic affinities. Concerning to biogeographic realms, Afrotropical (16.6%), Indomalayan (16.3%), and Neotropical (49.2%) are the biogeographic areas that harbour the highest percentage of species (Stuart *et al.* 2004). Meanwhile, in this global vision species diversity tends to be especially rich in habitats as moist Forests, tropical mountain regions, and flowing freshwater (Stuart *et al.* 2004; IUCN 2008; Hof *et al.* 2011). Such amphibian Hot spots, which usually contain high levels of endemism and diversity, are a particular concern for conservation (Seymour *et al.* 2001).

Also, the geographic distribution of declining species and extinctions worldwide is non-random.

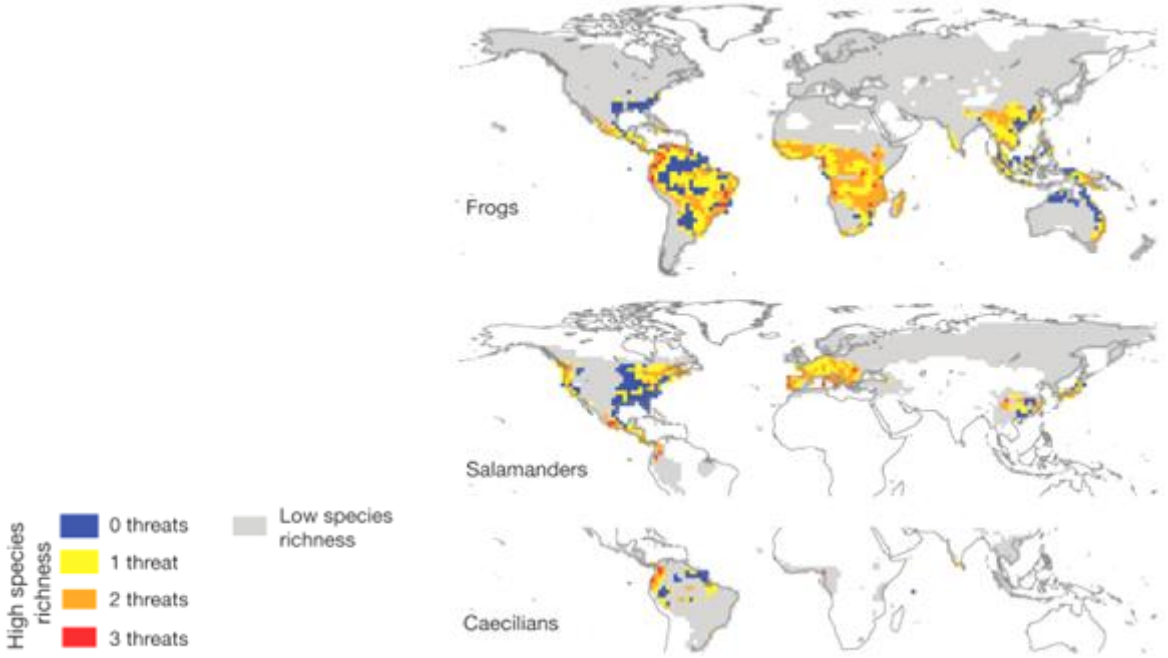

Figure 2 – Spatial overlap between areas with the highest amphibian species richness and the main factors threatening global amphibian diversity, projected for 2080 (Hof *et al.* 2011).

As previously referred, several factors have been pointed as responsible for the present amphibian decline crisis. Many studies have assessed how some threats affect amphibian populations and suggest that may exist a synergetic interaction between them. Climatic change can potentiate the emergence of opportunistic diseases (Daszac *et al.* 2003; Fisher 2007; Blaustein *et al.* 2010). Recently Luquet *et al*. (2012) showed that genetic erosion in amphibian populations due to, for example, low dispersal range or habitat fragmentation can decrease the capacity of population to cope with infectious disease emergence. In order to understand addictive threats to amphibians, *Hof et al.* (2011) used a bioclimatic envelope model to project climate change impacts on amphibian diversity and evaluated the addictive effects of pathogens, climate and land-use change for global amphibian diversity. The projected scenario for 2080 results (Fig.2) suggested that a risk assessment focused on a single threat are probably a more optimistic view, and an integrated evaluation, are effectively more realistic.

In order to avoid the underestimation of possible interactions, risk assessment combining the study of different environmental stressors must be carried out to make a more ecologically relevant evaluation of their potential effects.

Also, there are other preponderant factors that contribute to amphibian sensitivity: permeable skin, biphasic life-cycle with an aquatic and a terrestrial life stage and limited dispersal ability make this group of organisms particularly vulnerable to exposure to environmental perturbations either in water or in soil. Their skin physiology provides not only free flow of water but also the diffusion of chemicals or other substances present in the environment across the skin. Amphibian ecology and behavior also act as risk enhancer being responsible for their exposure to various sources and a variety of stressors (Bancroft *et al.* 2008).

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Structure and function of amphibian skin

The skin forms the major barrier separating the external surface of animals from their surrounding environments. However, while its major role in most vertebrates is to provide protection and insulation, in amphibians, skin is a permeable and physiologically active structure playing an important role of protection and homeostasis maintenance (e.g. uptake of water or excretion of harmful substances) (Wells 2007).

The amphibian skin has two components; the superficial epithelium or epidermis, and the underlying connective tissues of the dermis. Lack of horny scales or other protective structures, the epidermis has 5 to 7 layers including: stratum germinativum, stratum spinosum, stratum granulosum, and stratum corneum. The skin surface, in contact with external environment (stratum corneum), has cells differentiated as keratinocytes, which means they contain the cytoskeletal protein keratin, and is best developed in amphibians that spend most of their time on land (Lillywhite 2006). During the breeding season the males of anurans and urodeles develop nuptial pads on some digits of the forelimbs, to facilitate firm gripping of the females during matting.

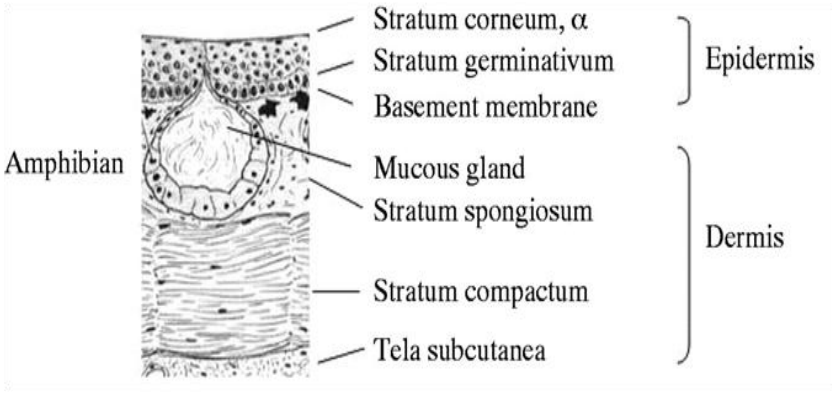

Figure 3 – Generalized features of amphibian integument. α indicate alpha-type keratin. (Lillywhite 2006).

This superficial layer is not permanent and molting is controlled by pituitary and thyroid glands (Wells 2007).

Generally, the skin of frogs and salamanders processes two types of multicellular glands mucous and serous (also known as granular or poison) glands. These glands are embedded in the dermis and open to the surface through connecting ducts. Mucous are responsible for the moist aspect of amphibian of outer skin surface, secretions prevent desiccation, facilitate temperature control, protect from abrasive damage and play an important role keeping the skin moist to facilitate cutaneous respiration (Lillywhite 1971; Daniels 2005). Serous glands are the primary source of chemicals active against predators and against invasion by microorganisms (Liu *et al.* 2013). As well amphibian integument differs along the body surface of individual and within species localization, abundance and secretions are also heterogeneous.

The dermis is two-layered, having an outer and looser stratum spongiosum and an inner stratum compactum. The stratum spongiosum is composed of a network of collagen and elastic fibers, along with a variety of specialized cells, chromatophores, glands, blood vessels, lymph spaces and nerves. Below this is the stratum compactum, consisting mainly of compact layers of collagen fibers (Wells 2007).

Pigmentation in amphibians is determinate by a functional unit of three types of chromatophores: xanthophores, iridophores and melanophores which are located in the dermis below the basal lamella. (Rudh 2013). As in many other animal groups, patterns of skin coloration play an important role on the behavior and ecology of amphibians (reviewed in Aspengren *et al.* 2009; Rudh 2013; Sköld 2013). Most significant aspects that were focused on previous works are: thermoregulation, water loss, camouflage to avoid predators or "background matching", aposematism and mimicry, communication and sexual display, and a response of environmental stress (Vences *et al.* 2002; Schmuck *et al.* 1988; Kobelt & Linsenmair 1992; Reviewed by Rudh 2013; Vásquez & Pfennig 2007; Hartmann *et al.* 2005; Kindermann *et al.* 2013). It is important to refer that physiological color change in amphibians is controlled mainly by hormonal pathways and is a relative slow response comparing with other animals (Aspengren *et al.* 2009; Sköld 2013).

In most amphibians, skin is also specialized as respiratory surface across which gas exchange occurs, with capillary beds in the lower epidermis and deeper dermis. In fact most amphibians depend largely on cutaneous respiration to meet their metabolic needs, and some, such as the lungless Family of salamanders use it exclusively. Gas exchange through the skin involves oxygen uptake from, and release of carbon dioxide into environment, but these exchanges are not necessarily of equal magnitude (Feder & Burgren 1985, Jared *et al.* 1999).

The outermost layer of amphibian skin, offer little resistance to water movement through the skin, making them subject to water loss via evaporative dehydration. Skin permeability to water differs throughout the body in either morphology or physiology. Different regions of the body varies in thickness of the various cell layers, vascularization, permeability to water and ions, e.g. some anuran species have a ventral specialized zone in pelvic region that due to the presence of cutaneous channels and high skin vascularization can take up water from a moist surface through the ventral skin (Wells 2007; Toledo *et al.* 1993). Beyond this, amphibians must sustain a hyperosmotic internal environment relative to the hypoosmotic external environment in order to maintain osmotic balance. This is realized using active regulation of transport of multiple electrolytes (including sodium, magnesium,

potassium, chloride) across the surface of the skin. A constant inward flux of exchange of sodium ions for potassium ions against an electrochemical gradient is accomplished via a cyclic AMP-regulated pathway. Water flow across the skin results when an osmotic gradient is established by electrical currents, induced by an exchange of these ions (Jørgensen 1997). A number of environmental variables can affect rates of ionic exchange between amphibians and environment. Acidic environments or diseases like the fungal infection chytridiomycosis can cause substantially increasing rates of sodium ion loss through the skin and inhibiting the active uptake from environments, what can cause fatal effects (Voyles *et al.* 2007; Campbell *et al.* 2012). High salt concentrations exposure is corrected by a cumulative process of urea in the plasma and tissues (Jørgensen 1997).

Amphibian Immune defenses

The immune system is an ensemble of biological structures and processes that when functioning properly protects the organism against disease. This system protects against infection as a layered component with defenses of increasing specificity (Mayer 2006) Some organisms possess exoskeleton or shells as examples of mechanical barriers that are the first line of defense against infection (Chitty & Raftery 2013). Amphibians carry a thin and delicate skin, lacking protection and highly permeable. Complementary, amphibian skin is permanently moist and mucus rich in glycoproteins, what suggests a good substrate for microbial or fungal development. In fact, there are cutaneous symbiotic bacteria which investigators attribute an increasing importance because of its metabolic products against pathogens. Chemical barriers also protect against infection. The skin serous glands are responsible for secreting antimicrobial peptides and enzymes that frequently display cytolytic activities (e.g. lysosyme) that can deter the growth of specific pathogens preventing or limiting infection (Rollins-Smith *et al.* 2002; Conlon 2011).

Innate immunity is accordingly to Richmond *et al.* (2009), "*First-line host defence that limits infection in the minutes or hours following pathogen exposure. These responses are nonspecific, do not confer long-lasting protection and consist of a limited repertoire of molecules. Innate immunity is phylogenetically conserved from insects to mammals, suggesting that the two systems arose from a common ancestor*." Authors have been considering antimicrobial peptides and the symbiotic bacteria as part of this innate immune system (Woodhams *et al.* 2007) along with, like all vertebrates, phagocytic cells, primarily macrophages and neutrophils that can directly phagocytize a pathogen.

Complement system that can kill bacteria directly by activation of the alternative pathways of membrane attack complex and natural killer (NK) cells are another element of the innate immune system of vertebrates. NK cells provide an immediate cytotoxic response against virus-infected or tumor targets in animals without previous immunization (Carey *et al.* 1999). Adaptive immunity system (lymphocite-mediated), the second layer of protection is activated by the innate response. Requires time to be activated, it is highly specific for a given pathogen and results in the generation of memory cells (Carey *et al.* 1999; Richmond *et al.* 2009). For example, frogs previously exposed to *Batrachochytridium dendrobatidis* (*Bd*) exposure are more resistant to secondary infections than frogs exposed for the first time (Richmond *et al.* 2009). Populations with disease history and persistence in the presence of *Bd* showed lower infection prevalence and intensity than those recently infected populations. Factors, as differences in skin peptides or symbiotic bacteria, have been pointed for differential response but adaptive immune response or environmental factors should be also included in these variations (Woodhams *et al.* 2007)

Evidence of skin microbial community skin and their growing importance

Symbiosis of microorganisms with invertebrate, vertebrate species (including man) or plants have been studied for decades in order to clarify the details of this relationship between host and symbiont. They affect a wide range of biological processes in the host as development, nutrition, defense against natural enemies and immunity (Bäckhed *et al.* 2005; Koren & Rosenberg 2006; Gage 2004). Obligate *versus* facultative relationships, endo- or ectosymbiosis are some of the aspects of this interaction that should be analyzed and somehow have importance for the host, the symbiont or both. Many other details of this relationship have been studied on the recent growth of research on symbiotic microorganisms as modes of transmission/ acquisition and the evolution host-symbiontic bacteria (Dale & Moran 2006).

The particular case of amphibians has special emphasis in the present work. Early, literature start reporting microbial presence in outer surface of amphibian skin but only recently with the amphibian mass decline and the emerging disease perspective, this thematic has been more intensely explored (Harris *et al.*2006; Beker & Harris 2010; Lauer *et al.* 2008; Walke *et al.* 2011). Antifungal cutaneous microbes have been isolated from every host species sampled, what suggests that they are widely present in amphibian

group. Also, growing evidences supports the hypothesis that antifungal bacteria play a role against various pathogens (e.g. chytridiomycosis or the fungus *Mariannaea* sp.) (Banning *et al.* 2008; Harris 2006). They produce antifungal metabolites that inhibit the fungal growth. Studies of bacteria removal from *Plethodon cinereus* skin demonstrated that individuals with reduce microbiota exhibited loss of body mass and higher morbidity than individuals with unmanipulated microbiota when exposed to *Bd* (Becker and Harris 2010). As well, it was reported that bioaugmentation of the skin of mountain yellow-legged frog, *Rana muscosa* with anti-*Bd* bacteria prevented morbidity and mortality comparing with those not treated with anti-*Bd* bacteria (Harris *et al.* 2009). Probiotic therapy through bioaugmentation is a feasible disease mitigation strategy suggested by many authors. (Woodhams *et al.* 2011; Muletz *et al.* 2012; reviewed by Bletz *et al.* 2013).

Many knowledge gaps still exist relatively to composition, mode of acquisition and specificity of these bacteria living on the amphibian skin surface. Recently, McKenzie *et al.* (2012) investigated three species of amphibians in different ponds hypothesizing that co-inhabit species with the same bacterial pool shared the same structure of cutaneous microbiome. Obtained results showed specificity between the symbiotic and host species. Individuals of the same species exhibited the same microbiome from population to population and, this specificity deferred between species. As well, population previously exposed to *Bd*, exhibited higher a percentage of anti-*Bd* bacteria in the skin and better response facing the infection, what suggests a sort of selection by the host (Woodhams *et al.* 2007).

Actually, the mechanism of microbial community acquisition, establishment or structure, still remains unclear. Analysis of culturable and unculturable bacterial species from the four-toed salamanders, *Hemidactylium scutatum,* indicates that bacteria associated with females and embryos within their nests were more similar to each other than either were to bacteria in the surrounding soil (Banning *et al.* 2008). At the same time, Walke *et al.* (2011) by analysing glass frog, *Hyalinobatrachium colymbiphyllum* adults and egg masses showed more evidences for vertical transmission of cutaneous bacteria. However, horizontal transmission has not been investigated in amphibians and environmental transmission has been demonstrated with *Pl. cinereus*, where the probiotic *Janthinobacterium lividum* was transmitted from soil to salamanders in a laboratory experiment, suggesting that effectively environmental transfer occurs in nature (Muletz *et al.* 2012). These modes of transmission of skin bacteria should not be seen as mutually

exclusive, neither the idea that they work isolated on the modulation of amphibian microbial communities.

Environmental conditions, skin properties and components or developmental stage of individuals are some of the biotic and abiotic factors that contribute and shape the amphibian skin microbiome. Despite amphibian skin is among the best-studied systems for understanding skin-associated microbial communities, most of the research is focused on the fungus *Batrachochytrium dendrobatidis*. Thus, knowledge is still in an embryonic stage and several gaps needs to be elucidated (Bletz *et al.* 2013).

Factors that could modulate amphibian resistance to pathogens

As mentioned above, amphibians have a well developed immune system. Some works were carried out in order to try to explain the gaps that may appear on immune defence mechanisms of these animals or the factors that can drive disturbance of homeostasis diverting the immune system from their normal functioning and may lead to its full decimation. Also, it should be noted that much of the found literature focuses mainly on infections related to chytridiomycosis, which was responsible for a high number of declines of amphibian populations (Carrey *et al.*1999; Pound *et al.* 2006; Daszac *et al.* 2003).

Natural factors such as changes in development or variations in body temperature of individuals has followed the evolution of amphibians, which does not explain the abrupt increase in the decline of amphibians due opportunistic infections. Some authors defend that these factors may act synergistically with other factors causing stress arising thereby and leading to an increased susceptibility to disease (Gahl & Calhoun 2010; Blaustein *et al.* 2010). Recently, Kueneman *et al.* (2013) showed the existence of a change in the skin microbial community in post-metamorphic individuals. This factor can help to clarify why the shift from larval to post-metamorphic life stage is accompanied by high mortality in amphibian and with severe instances of chytridiomycosis immediately following metamorphosis in some species. Moreover, ectoterms are strongly influenced by ambient temperature that also affects immunity system. For example, lower temperatures can affect antimicrobial peptides activity (Matutte *et al.* 2000). Prolonged cold, for instance, during hibernation can substantially decrease the immune response. However, the risk of disease is also dependent on the pathogen temperature dependence. Therefore, climatic changes can constitute a stressor, in sense that it can change the dynamic host-pathogen

and lead to an increased severity of infection. Changing the environment in which they are inserted, modifies the distribution, increasing the risk of transmission and even affect the ecology of populations leaving them more exposed and more susceptible to pathogens.

Ultra violet light exposure decreases the hatching success of many amphibian species. Moreover UV-light can interact synergistically with a variety of chemicals, low pH and pathogens (Kiesecker & Blaustein 1995; Long *et al.* 1995).

Exposure to metals and various man-made chemicals compromise immune function. They are a symptom of a general degradation of ecosystem by human activities. Christin *et al.* (2004) showed that exposition of *Xenopus laevis* and *Rana pipiens* for a short period of time to a mixture of pesticides can alter some aspects of immune response (e.g. phagocytic activity and lymphocyte proliferation). The idea of synergetic relations among different stress factors have been suggested as one possible reason for the emergence of opportunistic diseases that contribute to amphibian population declines. Other example is that pesticides increase the effectiveness of trematode infection (Rohr *et al.* 2008). Post metamorphic juvenile *Rana boylii* exposure to carbaryl, decrease recoverable peptide levels for at least 3 days and could potentially increase frog's susceptibility to chytrid (Davidson *et al.* 2007).

How environmental stressors act on the individual, how affect e.g. the antimicrobial peptides and how bacterial community can be affected are still standing a gap without elucidation and there is a high and urgent need to understand this complex process interactions in order to implement better amphibian conservation practices.

Objectives

Accordingly to the above mentioned, the two main objectives of this study were:

1) To characterize the bacterial community associated to the skin of the Perez´s frog (*Pelophylax perezi* Seoane) – inter and intra-population variability;
2) To evaluate the effects caused by exposure to chemical contamination – rich metal contaminated effluent – in the diversity of cutaneous bacterial community of *P. perezi*.

2. Study Areas and Methodology

2. Study Areas and Methodology

2.1. Study areas

To attain the objectives defined for this work, populations of the Perez' frog *Pelophylax perezi*, were sampled at five aquatic ecosystems, which were selected according with the presence/absence of chemical contamination. More than one reference site was chosen to account for natural variability caused by other factors rather than chemical contamination, and, thus allows establishing causality regarding chemical contamination and observed variation within populations. These five sampling sites were located along the Portuguese territory (Fig.4): Alto de Airão (AM), Salreu (SL), Vale de Açores (VA), Lagoa das Braças (LB) and Água Forte (TP).

Figure 4 - Map of Portugal with indication of the location of the five sampling sites.

Three of the sampling sites were freshwater and were considered as reference sites, one site was a water system characterized by fluctuations in salinity and the other one was a contaminated site with several metals (Table 1) (Luis *et al.* 2012; Maia *et al.* 2012).

Table 1- Information regarding coordinates, code and type of water for the five sampling sites.

Coordinates (Lat/ Long)	Code	Water
41°27'2 N/ 8°24'45 W	AM	Reference freshwater
40°43'57 N/ 8°34'20 W	SL	Reference (fluctuations in salinity)
40°26'5 N/ 8°15'53 W	VA	Reference freshwater
40°14'32 N/ 8°48'17 W	LB	Reference freshwater
37°57'31 N/ 8°14'2 W	TP	Contaminated with metals *

* Luis A. T., Novais M. H., Van De Vijver B., Almeida S. F. P., Ferreira Da Silva E. A., Hoffman L., *et al.* (2012). *Pinnularia aljustrelica* sp. nov. (Bacillariophyceae), a new diatom species found in the acidic waters in the Aljustrel mining area (Portugal) and further observations on the taxonomy and ecology of *P. acidophila* Hoffmann et Krammer and *P. acoricola* Hustedt. Fottea 12, 27–40.

Alto de Airão (AM in Table 1)

Figure 5 - Affluent of Rio Pele (Rio Ave watershed). Photo credits to Sara Costa.

This sampling site is located in northern of Portugal, integrated in the typical Minho landscape. With spring in a place commonly called Tanger, the brook became known as Ribeiro de Tanger, one of the small affluents of Rio Pele (Rio Ave watershed). The sampling point was situated a few meters downstream from the spring, in the Airão Santa Maria parish, (Guimarães), in a rural location, surrounded by small villages, fields of cattle, and goat grazing and small areas of subsistence farming.

Salreu (SL in Table 1)

Figure 6- BioRia, Salreu. Photo credits to Ricardo Carvalhal.

Situated in one of the most important wetlands of Portugal, the Ria de Aveiro, Salreu has rare habitat characteristic of Baixo Vouga Lagunar, designated *Bocage*. This landscape is formed through the use and control of freshwater habitats that span rivers, creeks and ditches. This creates conditions for agricultural practice, which include paddy fields and pasture in full harmony with the transitional habitats like marshes, reed beds and rushes. Besides its reticular mosaic landscape of undeniable value and where the marine influence is manifested daily through creeks that run through the area, this habitat has a high associated biodiversity (Brito 2006), like the: Purple Heron (*Ardea purpurea*), Otter (*Lutra lutra),* Common Tree Frog (*Hyla arborea*), Iberian Painted Frog (Discoglossus galganoi), Perez' frog (*Pelophylaz perezi*) are some of the species that could be found. However, this privileged natural area has some sources of environmental pollution: diffuse agricultural facilities and livestock, generate high loads of wastewater, rich in organic matter (Cerqueira *et al.* 2008).

Vale de Açores (VA in Table 1)

Figure 7 – Influent sampled in Vale de Açores, Mortágua. Photo credits to CM Mortágua.

This lotic system is a small water line located in Vale de Açores, Mortágua County. This inffluent is part of the Mondego watershed, running into the Aguieira dam. Surrounded by Caramulo and Buçaco saws, this area still retains some natural places, well preserved and almost pristine and presents some native forest species and diversity of animal species like wild

boar *Sus scrofa* L., fox *Vulpes vulpes*, and we can found in its clear waters a vast fish fauna, as bogas (*Iberochondrostoma lusitanicum)*, barbel (*Barbus* sp.) and trout (*Oncorhynchus* sp.) (http://www.cm-mortagua.pt/docs/Turismo/Pedestre.pdf).

Ribeira da Água Forte (TP in Table 1)

Figure 8 – Ribeira da Água Forte, Aljustrel. Photo credits for Sara Costa.

Ribeira da Água Forte is a stream located in the Aljustrel mining area in the Iberian Pyrite Belt. This water body drains the area of the old mining dumps, which have for decades been a source of acid waters and flows until its confluence With Ribeira do Roxo (Luis 2007). Contamination source for groundwater, streams, rivers and soils, acid water results from a process triggered by pyrite oxidation, mostly aided by bacterial activity, producing acid waters with very low pH, colloidal Fe and high concentrations of transition metals, which were important constituents in the exploited ores (Maia *et al.* 2012). We also can find explorations of iron oxide and manganese. Water presents a red-brown shade resulting from precipitation of dissolved iron particles. The vegetation in the area has become degraded or absent. In areas where it was possible to detect some tree and shrub formations, highlighted the presence of some gum rockrose (*Cistus ladanifer*) ruderal populations and exotic plantations, including eucalyptus (*Eucalyptus globules*), predominating over local native vegetation as holm oak (*Quercus rotundifolia*) *and* cork oak (*Quercus suber*). In addition to the weak diversity of plant structures, we also have low presence and low diversity of animal species (Venade 2005; Luis 2007).

Lagoa das Braças (LB)

Figure 9 – Lagoa das Braças, Figueira da Foz. Photo credits for Sara Costa.

Lagoa das Três Braças or simply Lagoa das Braças is a shallow freshwater coastal lagoon with about 28ha of extension situated in Figueira da Foz, Coimbra district. This lagoon integrates the Site "Dunas de Mira, Gândara e Gafanhas" and it has the status of Corine biotope (Martins et al 2006). This lagoon is fed by water from rainfall and underground water, however, it is partially dry in the summer (Oliveira 2006). The surrounding area is predominantly *Pinus* sp. wood forest. Water extraction for human consumption is currently used only in specific situations. The pond is located away from population clusters. Disturbing factors are the use of the pond for recreational purposes and the presence of the infesting *Myriophyllum aquaticum* L.. It is also an important local of shelter and nesting for some aquatic bird species and sporadically it could be found otter *Lutra lutra* L. traces.

2.2. Characterization of the target species

Pelophylax perezi

Perez's frog, as recently reclassified *Pelophylax perezi* López-Seoane 1885 is the most abundant amphibian that could be found in Portugal. This anuran is widespread and common over much of the Iberian Peninsula and South of France and has been introduced in some Balearic and Canary, Islands, Azores and United Kingdom (Loureiro *et al.* 2008). Its presence decreases with altitude (Almeida *et al.* 2001) but it has been reported expanding to higher elevations, possibly as a result of climate change (Bosch *et al.* 2009).

Considered an ecological generalist, the Perez' frog can inhabit a large variety of habitats, such as costal dunes, mountain, agricultural and forest zones, in a wide range of environmental conditions but it is conditioned by the existence of temporary and permanent water availability (both lotic and lentic waters), mainly during the reproduction periods.

Despite the fact that amphibians are well known as sensitive organisms, this species appears to be most tolerant to contaminants, exhibiting some resistance to extreme environments with some degree of pollution and salinity (Sillero & Ribeiro 2010; Marques *et al.* 2013).

Due to the species distribution, facility of sampling, general characteristics and absence of previous studies about the skin microbiome in *P. perezi* it was selected to carry out the present work.

2.3. Frog sampling and swab preservation

2.3.1. Sampling Perez's frogs

Ten adults were sampled from each of the five different sites. Between 5 and 7 samples from each of the five sites were used to perform density characterization and DGGE profiling. For bacterial colony isolation and purification, only were used samples from three sampling sites: Salreu and Lagoa das Braças with three swab samples analyzed and Aljustrel with five. The remaining sample swabs were preserved or used in protocol optimization. Each individual was manually captured with fishing net and handled with nitrile gloves previously disinfected with alcohol 70%. Then, the microbiome was collected and animals were maintained closed in a bucket until the end of sampling. Gloves were discarded after handling each individual. One liter of water was collected at each site for physical and chemical characterization.

2.3.2. Collecting microbiome / rinsing and swabbing frogs

Skin microbiome was immediately sampled at the field, after the capture of each individual. For this procedure, the protocol described by Brem *et al.* (2007) was adapted. Briefly, all the individuals were picked, using nitrile gloves disinfected with alcohol 70%, and they were rinsed abundantly on both sides (dorsal and ventral side) with sterile distilled water to ensure the collection of skin-associated microbes rather than pond-associated transient microbes.

Then, microbes were collected using sterile swabs that were passed along the length of ventral and dorsal region, head, lateral region, surface of tight, and foot. During the individual sampling, we rotated the swab, to make sure that all the sides of swab were impregnated and to avoid the saturation of only one area. This procedure allowed a more consistent sampling of the entire surface of the animal. This step was carried out by two researchers in order to prevent contamination and to minimize the handling time of the amphibians, to prevent desiccation and contact with another substrate. At the end, all the animals were released into their natural habitat. To preserve the samples, the swab were placed in a 1.5ml eppendorf tube with about 100µl of nutrient Broth (NB) solution (Nutrient Broth, Merck, Germany,Table 2) and immediately stored in thermal cuvettes with ice. As

soon reaching the laboratory, the samples were frozen and kept at -80 ºC until further use.

Table 2 - Typical composition of liquid nutrient broth (NB).

Nutrient Broth	Composition (g/L)
Peptone from meat	5
Meat extract	3

Immediately before the analysis, the frozen samples were thawing, and then one volume of 600µl of NB medium was added. Cotton was carefully unfolded from swab and the rest discarded. The cotton was intensely stirred with the vortex in order to suspend most of the cells attached to the cotton into solution and produce a homogenized suspension. Immediately after this procedure a volume of 100µl of previous solution was pipetted to an eppendorf tube with 500µl of NB medium with 15% glycerol. These samples were vortex to mix, frozen at -80°C, and used for growth cultivable fraction of bacteria present in the sample. The rest of the samples were reserved in stock for DNA extraction. The entire procedure was carried out in a flow chamber with flame.

2.4. Bacterial growth and isolation

2.4.1. Heterotrofic bacterial cultivation

The number of cultivable heterotrophic bacteria was determined using the low nutrient medium R2A (Oxoid, England, Table 3) inoculated with 100µl of sample suspension. The content pipetted on the plate was spread with a Drigalski sterile glass loop, according to the spread plate method until the spread liquid was absorbed by medium. The plates were incubated at 22°C for 8 days and the number of colonies counted after incubation.

2.4.2. Bacterial Density

Each bacterial cell, after its division will result in a colony, in other words means that is a colony forming unit (CFU). The number of bacterial colonies on each plate was counted to estimate the density of bacteria present in the skin of the frogs from different locations.

The count was made at the magnifier to estimate the amount of CFU per sample used the following formula:

(Dilution Factor x CFU)

Dilution factor for this work was estimated in 25.

Table 3 - Typical composition of liquid nutrient broth (NB).

R2A Agar	Typical Formula (g/l)
Yeast extract	0.5
Proteose peptone	0.5
Caseín hydrolysate	0.5
Glucose	0.5
Starch	0.5
Di-potassium phosphate	0.3
Magnesium sulphate	0.024
Sodium pyruvate	0.3
Agar	15

2.4.3. Bacterial colony isolation and purification

After counting, the plates were stored at 4°C for isolation of the different colony morphotypes strains. Colonies were chosen trying to represent all morphotypes present on the plates according to the observable morphological characteristics such as color, border, size, brightness and texture. The morphology of the cell of the selected colonies were observed under phase-contrast microscope in order to check if the microorganisms were bacteria, fungi or yeasts because only bacteria were targeted in this work.

In a flow chamber each isolated colony was transferred to a new plate with R2A medium with a sterile loop and spread. The cultures were incubated at 22°C and after 72 hours were observed to verify the presence or absence of contamination. Once again were

picked and transferred to new plate. After 4 days, the colony purity was checked again. Pure cultures were frozen at –80ºC in 1mL of NB-medium with 15% glycerol so we removed one loop 10µl of cells of the culture to a tube of freezing and reserved for later use.

Were only isolated bacteria from three different sites (Salreu, Braças and Aljustrel), and at a maximum of ten different colonies per frog sampled.

2.5. Identification of bacteria from frog skin

2.5.1. Nucleic acid extraction

Bacterial genomic DNA was obtained by Guanidina, EDTA and Sarcosil (GES) Method (Pitcher *et al.* 1989). This protocol is able to extract nucleic acid from Gram positive and Gram negative Bacteria.

Each isolated was subjected to DNA extraction. To chemically lyse the cells to release DNA, a sterilized 1µl loop full of cells was added in 100µl of Lysozyme solution (TE Buffer (Tris EDTA) with 25µl of lysozyme (100mg/ml) (Sigma)) and incubated at 37ºC overnight. Then 500µl of GES (Guanidine thiocyanate -EDTA-Sarkosyl) were added and the solution was vortexed to mix and incubated on ice 10 min. After, 250 µl of NH_4Ac 7.5M (ammonium acetate) were added, the solution homogenized and remained on ice for 10 min. After this period, 500µl of chloroform / ethanol isoamyl alcohol (24:1) were added to the solution. Then it was mixed by inversion and proceeded to centrifugation at 13 200 rpm for 20 min at room temperature. After centrifugation the supernatant was collected and transferred to a fresh tube to which were added again 500µl of chloroform / ethanol isoamyl alcohol (24:1), mixed, centrifuged and the supernatant was collected to a new tube. To precipitate DNA we used 600µl of cold isopropanol (Sigma, Madrid, Spain) and gentle mixed, followed by new centrifugation (13 200 rpm for 15 min a room temperature). The precipitated the DNA was washed with 150µl of cold 70% ethanol. After washing, the DNA obtained was dried due the evaporation of all ethanol. Finally, the obtained DNA was resuspended in 30µl of filtrated and sterilized MilliQ water.

2.5.2. 16S rRNA gene amplification

The amplification of the 16S rRNA gene of each isolate was performed by PCR technique. Each preparation was performed on ice, under sterility conditions, by using sterilized countertop and all the necessary material. To the PCR reaction mixtures, we used filter and sterilized MilliQ water (Milli-Q^{TM} system, Millipore, USA). DNA from bacterial isolates was amplified with universal bacteria primers 27F- 5'-AGAGTTTGATCMTGGCTCAG and 1525R - 5'-AAGGAGGTGWTCCARCC (Rainey *et al.*1996).

The PCR reaction mixtures were run in volumes of 30µ in the Thermo cycler (MJ Mini Bio-Rad Thermal Cycler). Amplification of the nearly full-length 16S rRNA gene sequence from each DNA was performed by PCR with primers 27F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1525R (5 ′-AGA AAG GAG GTG ATC CAG CC-3 ′) (Rainey *et al.* 1996). The 30µl PCR contained 2.1 µl 10x NH_4 Reaction Buffer, 0.9 µl 50mM $MgCl_2$ Solution, 6 µl dNTP's (1mM), 0.3 µl of each primer, 0.3 µl BioTAQ^{tm}DNA Polimerase (Bioline).The PCRs were run using the following conditions: an initial 3.5 min denaturation at 94ºC followed by 35 cycles of denaturation (1min at 94ºC), annealing (1 min at 55ºC) and extension (2 min at 72ºC) followed by a final extension of 10 min at 72ºC . After the end of the incubation program, PCR products were taken and immediately processed or stored at 4ºC for a time period as short as possible.

To check the success of PCR amplification of 16S rRNA, we used the final volume of the reaction for running on agarose gel stained with ethidium bromide. Agarose gel (1%) was prepared by boiling 1.2g of agarose in 120 ml of 1xTAE buffer (dilution of a 50xTAE stock solution, see Table 4). After cooling the solution, 8 µl of ethidium bromide were added and the agarose transferred to a gel container to polymerise. Then the DNA mixed 3µl of loading buffer was applied in gel wells, and 90-95 V were applied for about 40 min. Finally to see the DNA, gel was exposed to UV illumination at transluminator Gel Doc^{TM} XR System (Biorad, Hercules, EUA) and checked if the length of the DNA amplification fragment was 1500bp.

Table 4- TAE buffer 50X stock solution L^{-1} composition.

50xTAE	**L^{-1}**
Tris	242g
Glacial acetic acid	57.1mL
500mM EDTA (pH 8.0)	100mL
H_2O milliQ	To 1L

2.5.3. Gel extraction and purification

In a successful PCR, we have to recover the amplicons for sequencing. Carefully, with UV lights on, we excise the DNA fragment of interest with a blade, leaving a space between the different bands to prevent contamination and place the agar cubes in an eppendorf tube. To perform the purification we used the purification kit NZYGelpure (NZYTech, Portugal) according to the manufacturer instructions. Briefly 400 µl of Binding Buffer (XP2) were added to the tube which contained the agar cube and incubated until the gel is completely melted. The mixture was mixed and transferred to a HIBind DNA- Mini Column and centrifuge at 13.2 x 1000 rpm, 2 min. Next, we add 500 µl of SPW wash Buffer with ethanol and centrifuge again at maximum speed, 2 min. Finally, DNA was eluted with 30µl of Elution Buffer heated at 70 ºC, and centrifuge at maximum speed.

2.5.4. DNA sequencing of PCR products from bacterial pure cultures and sequence analysis

DNA sequencing of bacterial isolates was performed at Macrogen facilities (Macrogen Inc., Korea). Sequences were read using the software Sequence Scanner v1.0 (Applied Biosystems). Their quality was checked and saved in fasta format with MEGA 5.1. All sequences were compared with sequences available in the GenBank database using BLAST network services (Benson *et al.* 1999). EzBIOCloud Bioinformatics program was also used to compare the isolates with reference prokaryotic 16S rRNA gene sequences in the database (http://eztaxone.ezbiocloud.net/ Kim *et al.* (2012).

Sequence alignments and phylogenetic trees were performed with MEGA5.1 editor (Tamura *et al.* 2011) using ClustalW on the basis of the neighbor joining method to estimate a simple tree and using maximum composite likelihood model.

2.6. Denaturing Gradient Gel Electrophoresis (DGGE) analysis of whole DNA from the outer microbiome of the frogs

2.6.1. Total DNA Extraction and Purification

Each total microbiome sample was homogenized and the cotton was carefully discarded. Total microbial genomic DNA was extracted and purified using the E.Z.N.A. ™ Soil DNA kit (Omega Bio-Tek Inc., Norcross, GA, USA) according to the protocol with some modifications: Step 1. Weigh 200 mg of glass beads to the sample with 1 ml Buffer SLX Mlus. Vortex at maximum speed for 3 minutes to lyse samples. Step 3. Incubate at 70°C for 10 min. Briefly vortex the tube once during the incubation and increase the temperature to 95°C during 3-5 min. Step 7. Carefully transfer supernatant to a new 2 ml tube and add 0.7 volume of isopropanol. Mix by inverting tube for 20-30 times. Always incubate the sample at -20°C for 1h.

All DNA was stored at −20°C before use.

2.6.2. DNA Amplification for DGGE

The V3-V5 region of the 16S rRNA genes was amplified with bacteria-specific primers 341F and 907R. Then, a Nested PCR was performed with primers 341F and 518R to amplify only the V3. A GC-clamp was applied to the 5′ end of the forward primer to increase the sensitivity of the DGGE analysis (Muyzer *et al.* 1993).
The PCR mixture comprised 3 µL of 10 × PCR Reaction buffer, 6 µL of 1 mmol− 1 dNTP mixture, 2.4 µL of 50Mm MgCl2, 0.3 µL of each primer (1 mmol− 1), 1.5 U Supreme NZYTaq DNA polymerase (NZYtech), 3 µL of DNA extracted from total skin microbiome template, and sterile water to a final volume of 30 µL.

Table 5 - Primer sets used for PCR of 16S rRNA genes for DGGE analysis of bacterial community of Perez's frog skin.

Primer	Sequence (5' → 3')	16S variable regions	Reference
341F 907R	CCT ACG GGA GGC AGC AG CCG TCA ATT CMT TTG AGT TT	V3-V5	(Muyzer *et al.* 1998)
341F* 518R	CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	V3	(Muyzer *et al.* 1993)
GC-Clamp	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G		(Muyzer *et al.* 1993)

[1]An "*" indicates that a GC-clamp was attached to the 5' end of this primer.

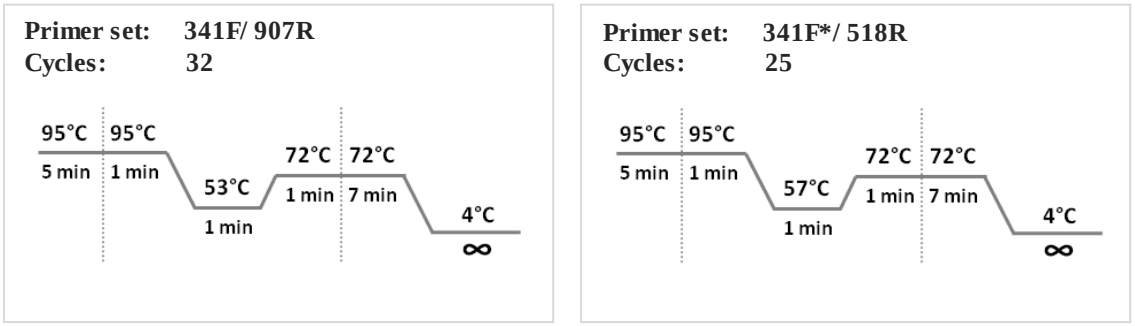

Figure 10 – Temperature cycle, primer set and number of cycles used in the PCR reaction ([1]An "*" indicates that a GC-clamp was attached to the 5' end of this primer.)

2.6.3. DGGE Protocol

The DGGE was performed according to the method described by Muyzer *et al.* (1993), using the DCode Mutation Detection System (BioRad, Hercules, CA, United States). The preparation of DGGE gels involves the following steps: solution preparation, apparatus cleaning and assembly, gradient casting and polymerization, sample loading and electrophoresis, staining, and gel imaging (Green *et al.* 2009).

1. *Solution preparation and DGGE running buffer*

The desired "high" and 'low" denaturing stock solutions were prepared according to Table 6 and then stock solutions were degassed for 15 minutes and stored protected from light at 4°C.

After, the required volume of 1 x TAE buffer for the DGGE system being used (approximately 8L) was prepared as well as the 10% ammonium persulfate (APS) prepared fresh daily by combining 1mL of water with 0.1 g of ammonium persulfate.

Table 6 - Denaturant acrylamide solutions used on DGGE.

Reagents	Example stock concentration and reagent volumes[1]		
	0%	30%	70%
TAE (50X)	2mL	2mL	2mL
Acrylamide (40%; 37.5:1)(BioRad)	20mL	20mL	20mL
Formamide (Sigma)	-	12mL	28mL
Urea	-	12.6g	29.4g
MilliQ Water	To 100mL	To 100mL	To 100mL

2. Apparatus cleaning and assembly

All plates and spacers were clean and dry before assembly. Glass plates, spacers and clamps perfectly align were correctly positioned, attaching the clamps to the glass plate and finally aligning spacers in the sandwich assembly, with the background sponge previously saturated in water.

3. Gradient casting and polymerization

The gradient maker was washed with distilled water and ensured that the tubing, channel between the two chambers of the gradient maker, and the needle were free of water and blockages by running the peristaltic pump. All the channels were closed. For each acryl high and low solutions we used a volume of 14mL of high (70%) and low (30%) acryl solutions and added 130 µl APS and 13 µl TEMED in both and mixed well immediately. Once APS and TEMED were added to the polyacrylamide solution, the polymerization reaction will begin. We put the solutions on the correspondent gradient maker chambers and turned on the pump. The solutions were mixing and the gradient was being formed during this process. We had to stop the flow when the acryl is approximately 0.5cm under the comb level. After pouring, a few ml of propanol were put on top to obtain a straight surface and waited 90 min to polymerize.

Next we cleaned the propanol with distillated water and filled up the gel chamber with a mixture of 5 ml acryl 0%, 100µl APS and 10 µl TEMED using a micropipette and placed the comb. Finally, we allowed the stacking gel to polymerize at least 30 min.

4. Sample loading and Electrophoresis

The comb was gently removed and the sandwich assembly attached to the core. Then we placed the core and attached gel assemblies into the buffer chamber, previously heated to 60°C. The samples were loaded into the wells with 3µ of loading buffer to each sample. Connected the power cord, and turned the power and heat on until the initial temperature. With the pump on gel was running for 17h at 70 V.

5. Post-electrophoresis staining and gel imaging

The pump and the power were turned off. Gel was removed from glass plates and incubated in 250 ml TAE with 15µl ethidium bromide during 30 min and gentle shacked.

Gel (Figure 17) was observed on the UV illuminator at transluminator Gel Doc™ XR System (Biorad, Hercules, EUA).

2.6.4. DGGE profile analysis

The profiles obtained in the DGGE gel were analyzed with Quantity One 1-D Analysis Software. Bands were marked and matched by hand and a cluster analysis was performed using UPGMA method (Figure 18).

2.7. Assess the tolerance of bacteria to metal contamination: toxicity assays

In order to understand the effects of wastewater contaminated with metals from Ribeira da Água Forte, Aljustrel on the outer microbiome of the frogs, we performed a toxicological assay with 30 bacterial isolates from the sites SL, LB and TP, genetically identified in 2.4.

Experimental conditions

The bacterial isolates on point 2.4 were thawed and placed in growth in LB medium for 8 days at 22°C. The metal-contaminated effluent water was kept frozen until used to ensure lower modification of the properties before testing. Bacteria were exposed to the following different effluent dilutions: 25%, 50%, 100% and the control.

Each bacterial isolate was suspended in liquid LB medium, previously autoclaved, until the Optical density of 0.4, measured with a spectrophotometer at a wavelength of 600nm (Jenway 6405 UV Spectrophotometer, Keison International Ltd).
To make up the different concentrations of effluent, metal contaminated water was diluted in autoclaved MilliQ water. Then to each dilution of the effluent were added 5µl suspension of each bacterium and gently mixed. Every sample incubated for 1h at room temperature, as a direct exposure to the chemical stressor. A volume of 100µl of each mixture were spread in Petri dishes with solid LB medium in triplicate for each concentration and placed at 22°C for 5 days. Growth was monitored every 24 hours using the following sign codes: (-) for growth absence and (s) for presence of growth, following by a (+) according with the density of colonies comparing with control until all the plate were covered.

Statistical analysis

For this assay, since we tested different bacteria species and we do not have a quantitative way of quantification of effects, we proceeded with a qualitative evaluation of the effects caused to chemical exposition based on resistence and specific growth rate (µ) in the presence of the toxic stressor. During the assay a symbol scale (see table 7) was attributed, characterizing bacterial growth in comparison with the corresponding control. For statistic analysis symbol scale was transformed in a growth category, in order of quantify the growth level and contamination sensibility (table 7).

Table 7 - Symbolic scale and growth categorization used during the performance of the ecotoxicity assays, in the assessment of the tolerance of bacteria to metal contamination.

Symbol	Growth Category	Meaning
-	0	Growth absence
s	1	<5 CFU
s+	2	>5 CFU
s++	3	An half of plate surface covered
s+++	4	Plate surface covered

2.8. Chemical characterization of the water

In each of the five sites where frog's population of *Pelophylax perezi* were sampled, about 1L of water was collected for posterior physico-chemical analysis (see table 18). Water was frozen and kept in the dark, from the time of collection until analysis. Frozen samples were thawed slowly and well-mixed before analysis. In addition, for the analysis of total and dissolved trace metal preservation, nitric acid (HNO_3) was added until waters' to pH <2 (Nitric acid ≥ 65%, SIGMA-ALDRICH Co.). Acidification decreased the precipitation of Fe, Cu, Ni, Al, and Zn from water samples (Wilson 1974). Several physico-chemical parameters of water were measured in the field during the collection: dissolved oxygen (OXI 330/SET, best nr. 200 232), pH (pH 330/SET-2, best nr. 100 788), conductivity (LF 330/SET, best nr. 300 204).

Total metal concentrations were analyzed using ICA (Central Analysis Laboratory of Aveiro University) facilities. Al, As, Cr, Cd, Cu, Fe, Pb and Zn concentrations were determined by the analytic method ICP-MS, according to the ISO 17294 norm. Other physico-chemical determinations (turbidity, SST, BDO_5, nitrites, nitrates, ammonia, calcium, magnesium and phosphorus) were performed using Hach DR/2000 protocols and reagents (Hach Lange Lda, Portugal) and salinity (LF 330/SET, best nr. 300 204).

3. Results

3. Results

3.1. Physical and chemical characterization of water samples

The characterization of physical and chemical parameters of water samples revealed low levels of dissolved oxygen at SL and TP, indicating a situation of anoxia in SL as values were below 1mg/L in the water column (Table 8). As well, these two sites showed the highest values of BOD_5: 26.4 mg/L and 24 mg/L, respectively. SL and TP also exhibited the highest values of salinity and conductivity: 3 and 1, 5720 and 2210 µS/cm, respectively. These high values in SL are associated with an increased salinization problem in Baixo Vouga, while at TP are related with metal contamination. TP also presents low pH values (pH= 4.93) and high concentration of metal, particularly Fe (48300 µg/L). TP study area is located in the Aljustrel mining region and is strongly affected by drainage from mine dumps, old cementation pools and the acid–water pond that forms in the tailings site. Acid-mine waters flow along the Ribeira da Água Forte and are mixed with reduced organic-rich waste waters in their path (Maia *et al.*2012). Regarding to particulate matter and consequent reduction in water transparency, two parameters were used: turbidity and TSS. High values were reported in SL and TP, 13mg / L and 113mg / L of solid suspended matter to SL and TP respectively. Mining and agriculture can lead to high sediment levels entering water bodies. Also, mining industries can generate very high levels of turbidity from colloidal rock particles. The systems AM and VA showed the lowest values for this parameters. SL and TP showed the highest values for Ca^{2+} and Mg^{2+} dissolved in water (SL: 328 mg/L and 96 mg/L; TP: 103 mg/L and 31 mg/L respectively). The geomorphologic characteristics in TP can influence the chemical composition of the water systems. Also sea water influence can contribute for the high concentration o these ions in SL. Nitrogen sources in natural waters are diverse. All water samples analyzed presented very low concentration of nitrite and nitrate forms. AM and VA recorded the highest values of nitrate (6.6 mg/L and 4.2 mg/L). Ammonia is present in considerable amounts in SL and TP (1.06 mg/L and 5.6 mg/L). These high values are associated to deterioration processes of waste materials of plant or animal origin, over-fertilisation. Furthermore, SL and TP also present the highest concentrations of phosphates (reactive phosphorus), 0.43 mg/L and 0.42 mg/L accordingly.

Table 8 - Values of physico-chemical parameters measured in the water column of the sample sites where *Pelophylax perezi* were collected and swabbed. (TSS: Total Suspended Solids; BOD_5: Biochemical Oxygen Demand over five-days**)**

Physico-chemical parameters	Sample sites				
	Alto Airão (AM)	Lagoa Braças (LB)	Salreu (SL)	Agua Forte (TP)	Vale Açores (VA)
Dissolved oxygen (mg/L)	10.3	7.5-9.8[a]	0.6	3.6	-
Conductivity (µS/cm)	124.8	293.0	5720	2210	116.7
pH	6.49	8.81	7.66	4.93	8.16
Salinity	0	0	3	1	0
Turbidity (FTU)	4	31	37	73	1
TSS (mg/L)	0	9	13	113	0
BOD_5 (mg/L)	2.09	1.04	26.4	24	1.64
Nitrites (mg/L NO^-_2)	0.013	0.07	0.05	0.008	0.016
Nitrates (mg/L NO^-_3)	6.6	0.4	0	0.3	4.2
Nitrogen, Ammonia (mg/L NH_3-N)	0.03	0.27	1.06	5.7	0.15
Ca^{2+} (mg/L)	2.08	16	328	103	5.6
Mg^{2+} (mg/L)	0.68	5	96	31	1.8
Phosphorus, Reactive (mg/L PO_4^{3-})	0.13	0.04	0.43	0.41	0.01
Al (µg/L)	32	75	43	333	16
As (µg/L)	< 0.50	16	5.4	87	0.51
Cd (µg/L)	0.21	0.31	0.05	0.84	0.07
Cu (µg/L)	1.5	5.2	2.1	148	1.6
Cr (µg/L)	0.55	2.8	1.0	1.6	0.76
Fe (µg/L)	19	5696	2976	48300	160
Ni (µg/L)	< 0.25	1.8	4.0	16	1.7
Pb (µg/L)	0.20	0.59	0.95	19	0.39
Zn (µg/L)	4.1	22	9.2	575	7.9

[a] Calado, 1993; Data relating to May-July de 1989

"-" measurement information not available for water column.

3.2. Density of cultivable bacteria on *Pelophylax perezi* frogs

The density of cultivable heterotrophic bacteria in the frog skin ranged from 150 to 7700 colony forming units (CFU) per frog swab sample. Significant differences were observed, among sampled *P. perezi* populations, on the measured average densities of skin bacteria (Kruskal-Wallis: p=0.0061).

When analyzing the average bacterial CFU per frog sample, frog's population from TP showed the lowest concentration of skin bacteria comparing with frogs from the remaining sampling sites. Conversely, SL and AM frog populations registered, on average, the highest density of bacterial CFU per sampled frog (Figure 11). However, significant differences were only registered between SL and TP, being the values of bacteria CFU density significantly higher in SL comparatively to TP (Dunn's test: $p<0.05$) (Table 9).

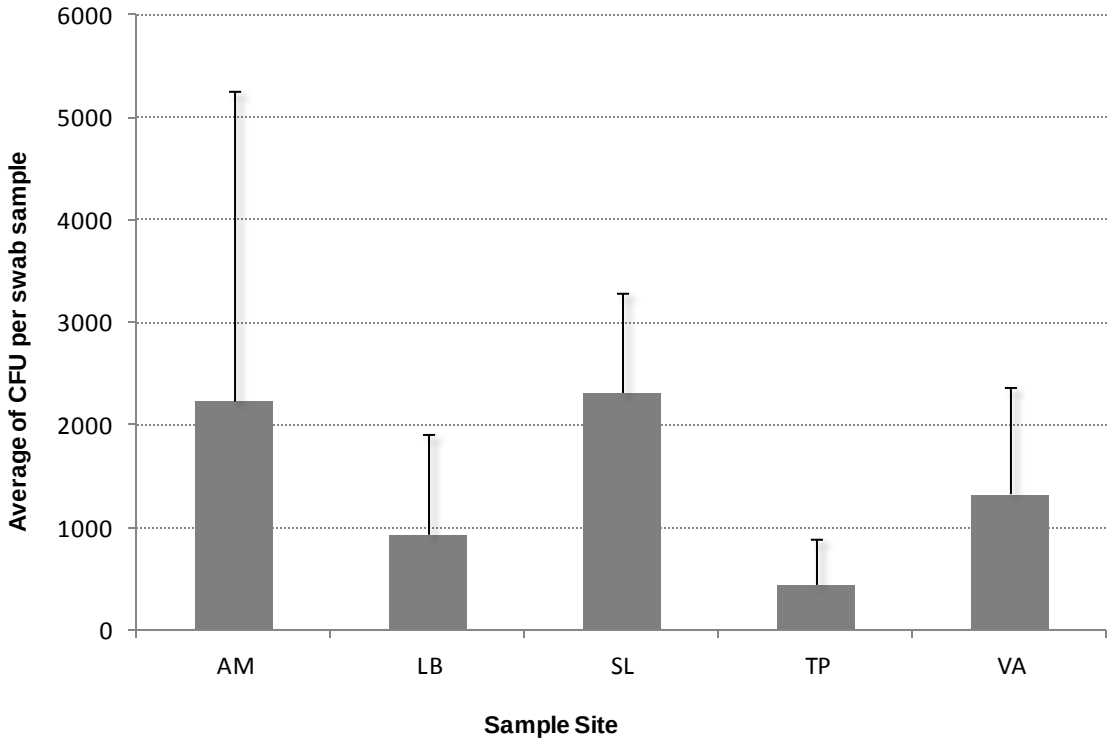

Figure 11- Bacterial densities of colony forming units (CFU) per individual swab sample from *Pelophylax perezi* populations sampled on this work.

Table 9 – Analysis of morphological distinct CFU's per sample site. (N – number of individuals sampled; Range – lowest and highest number of different CFU's; SD – standard deviation). Letters a and b correspond to homogenous groups accordingly to Dunn's test results.

Sample Site	N	Range	Average	SD	Homogeneous groups
Alto de Airão (AM)	6	4 - 10	6.67	2.50	a,b
Lagoa Braças (LB)	7	2 - 10	6.24	1.08	a,b
Salreu (SL)	7	7 - 11	9.14	0.63	a
Agua Forte (TP)	5	3 - 6	5.20	0.58	b
Vale Açores (VA)	6	5 - 10	8.5	1.98	a,b

On average, adult individuals of *P. perezi* harbored approximately between five and nine morphological distinct CFU's (Table 9). Concerning to the quantity of morphological different colonies, SL showed the highest average and TP the lowest average value.

3.3. Diversity of the cultivable bacterial community

To estimate the diversity of cultivable bacterial community, 74 different bacterial strains were isolated and identified from 11 different frogs: 5 individuals from TP, 3 from SL and 3 from LB. In total 23 bacterial species were identified in SL sampled frogs, 24 in TP frog's and 27 in LB.

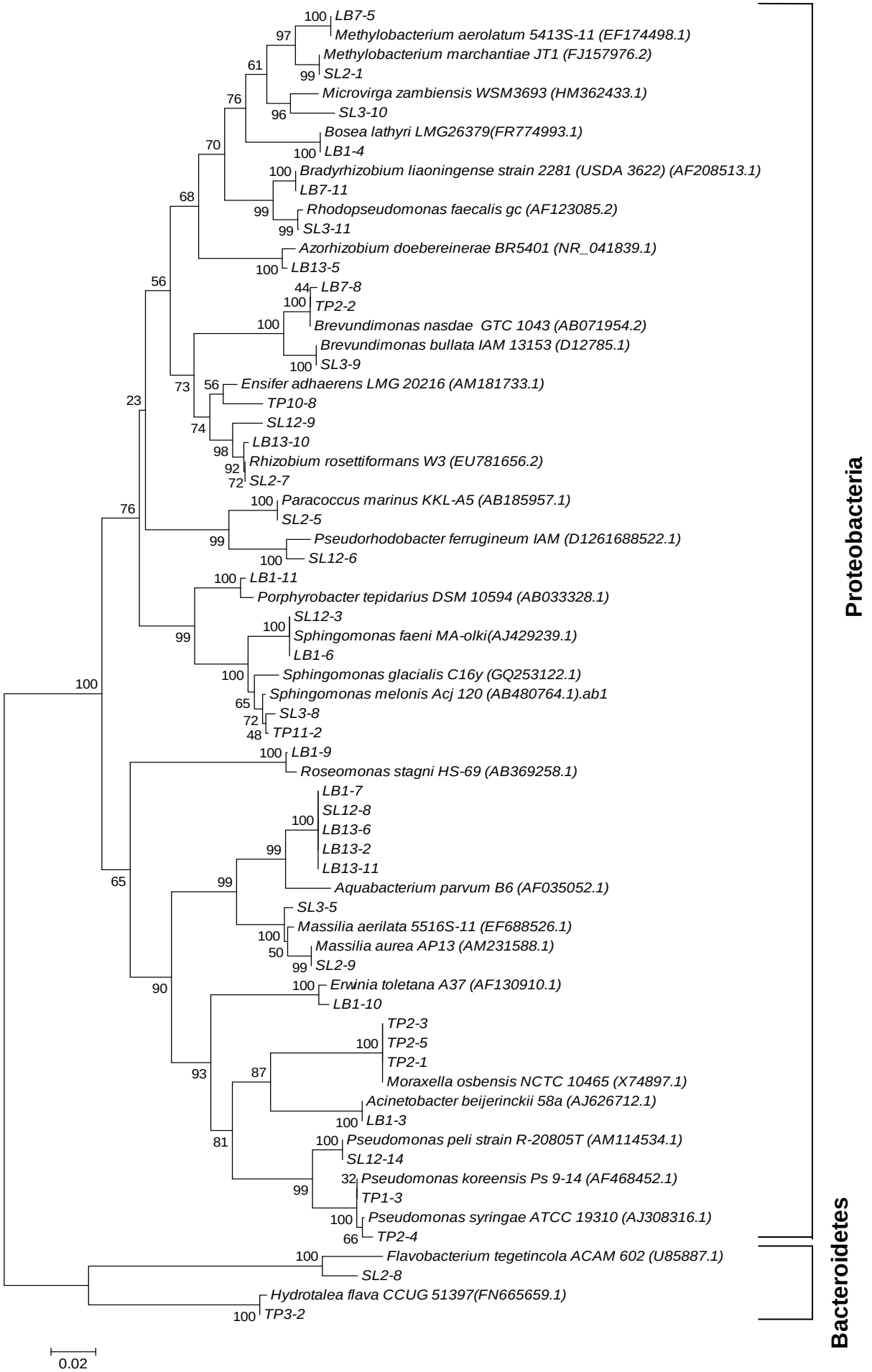

Figure 12 - Phylogenetic tree based on 16S rRNA gene sequences from the bacterial isolates belonging to *Proteobacteria* and *Bacteroidetes* Phyla, together with reference Taxa. Tree was determinate by neighbor-joining analysis with a bootstrap support (1000 replicates).

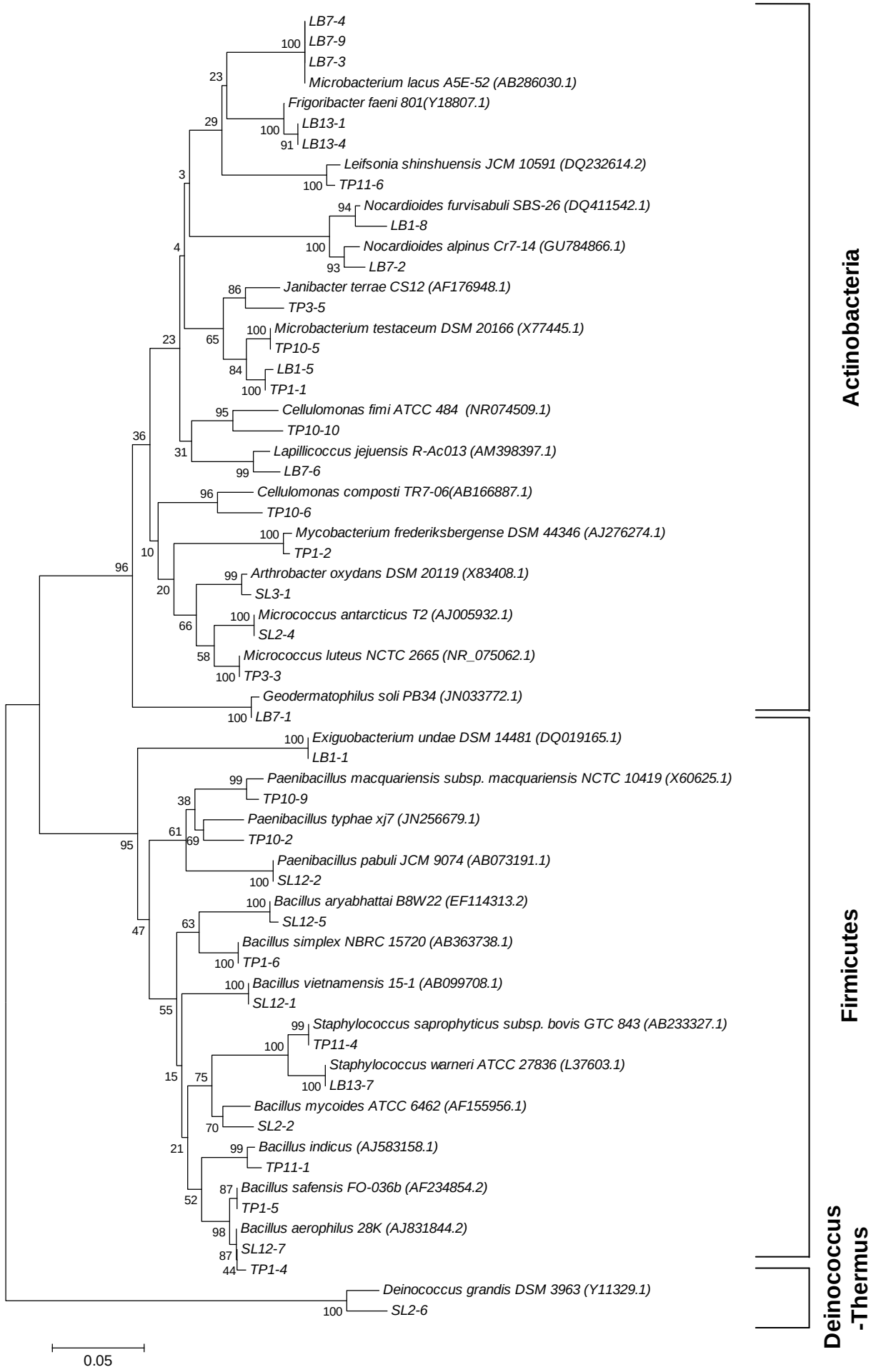

Figure 13 - Phylogenetic tree based on 16S rRNA gene sequences from the bacterial isolates belonging to *Actinobacteria, Firmicutes* and *Deinococcus-Thermus* Phyla, together with reference Taxa. Tree was determinate by neighbor-joining analysis with a bootstrap support (1000 replicates).

Analysis of the 16S rRNA gene sequences of the isolates identified five Phyla within the domain Bacteria: *Proteobacteria, Actinobacteria, Firmicutes, Deinococci-Thermus* and *Bacteroidetes*. Isolates belonging to the Phylum *Proteobacteria* were dominant in all the three studied frog populations. Frogs from TP and LB had *Actinobacteria* as the second more common Phylum. Conversely, the bacterial skin samples of *P. perezi* from LB population did not include *Deinococci-Thermus* and *Bacteroidetes* isolates (Figure 14).

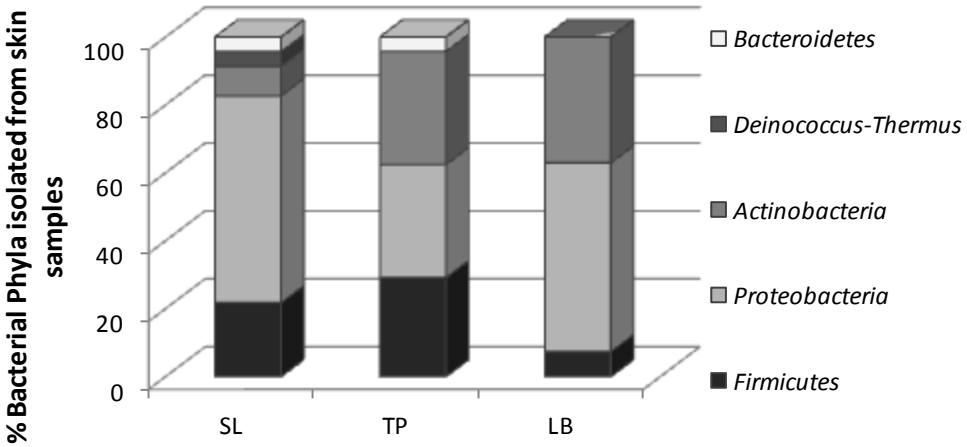

Figure 14- Microbial diversity of bacteria at Phyla level of partial 16S rRNA gene sequence from isolated bacteria from skin samples of three populations of Perez' frog *Pelophylax perezi* studied: Salreu (SL), Água Forte (TP) and Lagoa das Braças (LB) determined by cultivation.

The majority of the identified isolates belonged to the Class *Actinobacteria* and *Alphaproteobacteria* (see Figure 15). The population of Perez's frog from LB had no isolates from the Classes *Deinococci*, *Flavobacteriia* and *Sphingobacteriia.* This population also presented the lowest percentage of bacterial isolates on the *Bacilli* group, contrary to the other analyzed frog populations. Isolates from the Classes *Flavobacteria* and *Deinococci* were found exclusively on the SL frog population, though *Sphingobacteriia* was found in TP (Figure 15).

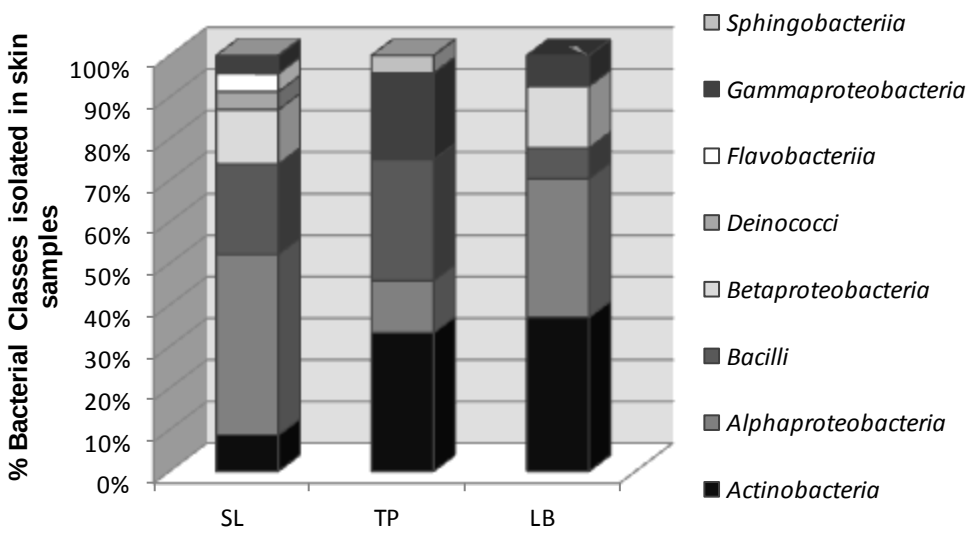

Figure 15- Microbial diversity of bacteria at Class level of partial 16S rRNA gene sequence from isolated bacteria from skin samples of three populations of Perez' frog *Pelophylax perezi* studied: Salreu (SL), Água Forte (TP) and Lagoa das Braças (LB) determined by cultivation.

When the diversity of bacterial isolates was evaluated at the Genus level, the number of isolates belonging to the different genera was low within the Classes *Betaproteobacteria* (Two Genera: *Aquabacterium, and Massilia*), *Bacilli* (four Genera: *Paenibacillus, Bacillus, Staphylococcus,* and *Exiguobacterium*), *Gammaproteobacteria* (four Genera: *Acinetobacter, Erwinia, Moraxella* and *Pseudomonas*), *Deinococcus* (one Genus: *Deinococcus), Flavobacteriia* (one Genus: *Flavobacterium), Sphingobacteriia* (one Genus: *Hydrotalea*) (see Figure 16). On the other hand, the Classes *Alphaproteobacteria* and *Actinobacteria* included a larger number of isolates belonging to many Genera and when these domains were present, they were substantially dominant. The Class *Alphaproteobacteria* was represented by 14 Genera: *Azorhizobium, Bosea, Bradyrhizobium, Brevundimonas, Ensifer, Methylobacterium, Microvirga, Paracoccus, Porphyrobacter, Pseudorhodobacter, Rhizobium, Rhodopseudomonas, Roseomonas* and *Sphingomonas.* The Class *Actinobacteria* was represented by isolates belonging to 11 Genera: Arthrobacter, *Cellulomonas, Frigoribacterium, Geodermatophilus, Janibacter, Lapillicoccus, Leifsonia, Microbacterium, Micrococcus, Mycobacterium* and *Nocardioides.*

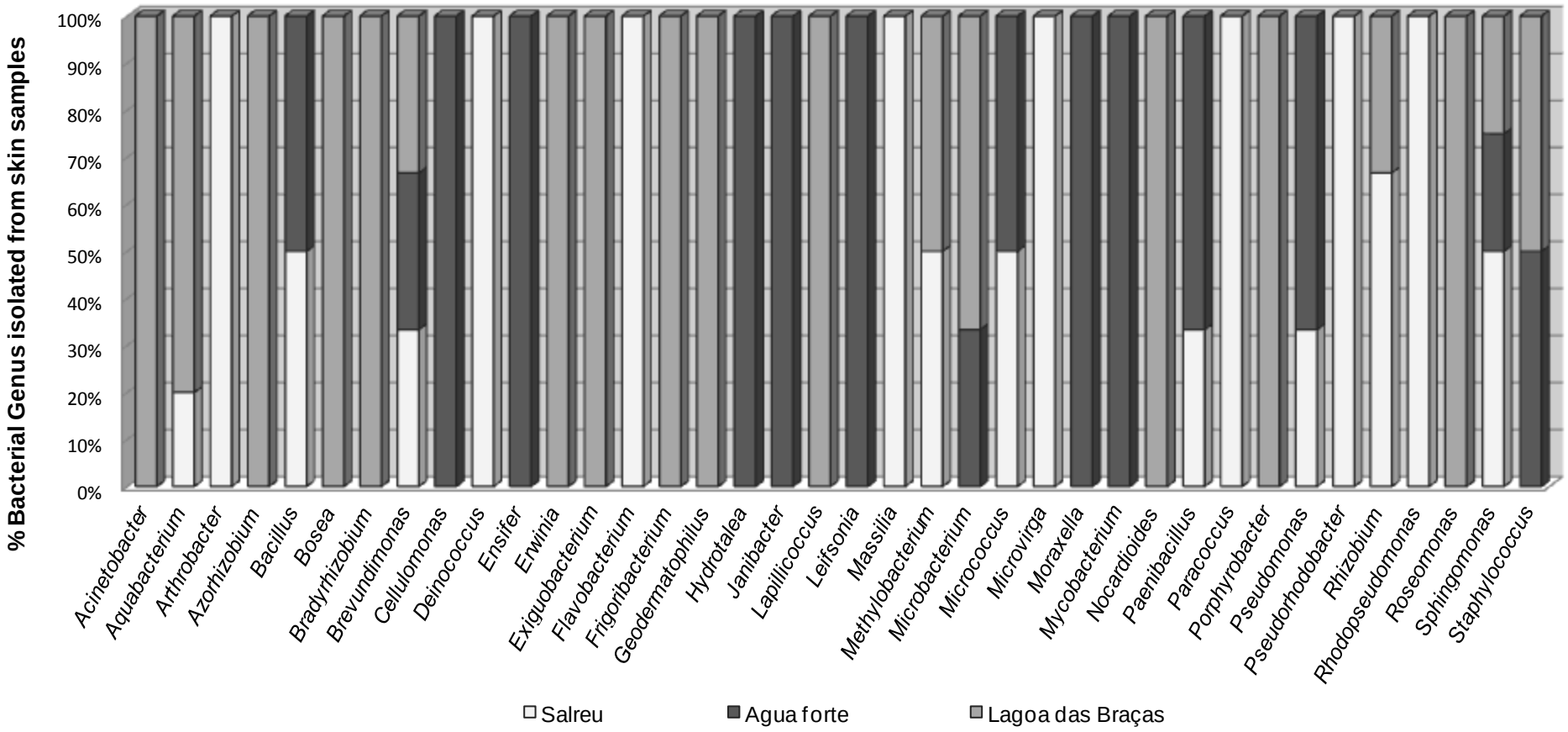

Figure 16- Microbial diversity of bacteria at Genus level of partial 16S rRNA gene sequence from isolated bacteria from skin samples of three populations of Perez' frog *Pelophylax perezi* studied: Salreu (SL), Água Forte (TP) and Lagoa das Braças (LB) determined by cultivation.

Strains from the Genera *Acinetobacter*, *Azorhizobium*, *Bosea*, *Bradyrhizobium*, *Erwinia*, *Exiguobacterium*, *Frigoribacterium*, *Geodermatophilus*, *Lapillicoccus*, *Nocardioides*, *Porphyrobacter* and *Roseomonas* were only isolated in frogs from the LB population. Strains from the Genera *Arthrobacter*, *Deinococcus*, *Flavobacterium*, *Massilia*, *Microvirga*, *Paracoccus*, *Pseudorhodobacter* and *Rhodopseudomonas* were only isolated from SL population and strains from the Genera *Cellulomonas*, *Ensifer*, *Hydrotalea*, *Janibacter*, *Leifsonia*, *Moraxella* and *Mycobacterium* were only isolated in the population from TP, the metal contaminated site (see Figure 16).

Only strains of the Genera *Brevundimonas* and *Sphingomonas* (Class *Alphaproteobacteria*) were found in all frog populations studied. When evaluated at the Genus level, Perez' frog population from TP showed the lowest number of skin cultivable bacteria (*n*= 24), with a low diversity, covering 15 different Genera (Figure 16). It was the only place where two of five sampled individuals had all the CFU isolated and identified. TP1 (n=6) had 3 *Bacillus*, 1 *Microbacterium*, 1 *Pseudomonas* and 1 *Mycobacterium*; TP2 (n=5) had 3 *Moraxella*, 1 *Brevundimonas* and 1 *Pseudomonas*. On the other hand LB population represent low microbial diversity of bacteria at the Phyla level but showed the highest diversity in Genera (19 different Genera represented).

Blast analysis of the 16S rRNA gene sequences of isolates evidenced two strains that showed < 97% similarities with their closest relatives; one belonged to the Genus *Aquabacterium* and another one to the Genus *Flavobacterium.* This result suggests that some of the sequences may represent new species.

3.4. DGGE of PCR product analysis from *P. perezi* swab samples

DGGE patterns from prokaryotic DNA in all frog skin samples have some heterogeneity and showed different phylotypes (Figure 17). Nevertheless, there are bands repeated in several samples belonging to frogs from the same sampling localization or from other different population, i.e., at least part of the microbial community could be common to frogs from different sites.

The total number of DGGE band positions detected in the gel was 32 and the number of DGGE bands per sample varied between 5 to 21 bands, being rather stable in all samples.

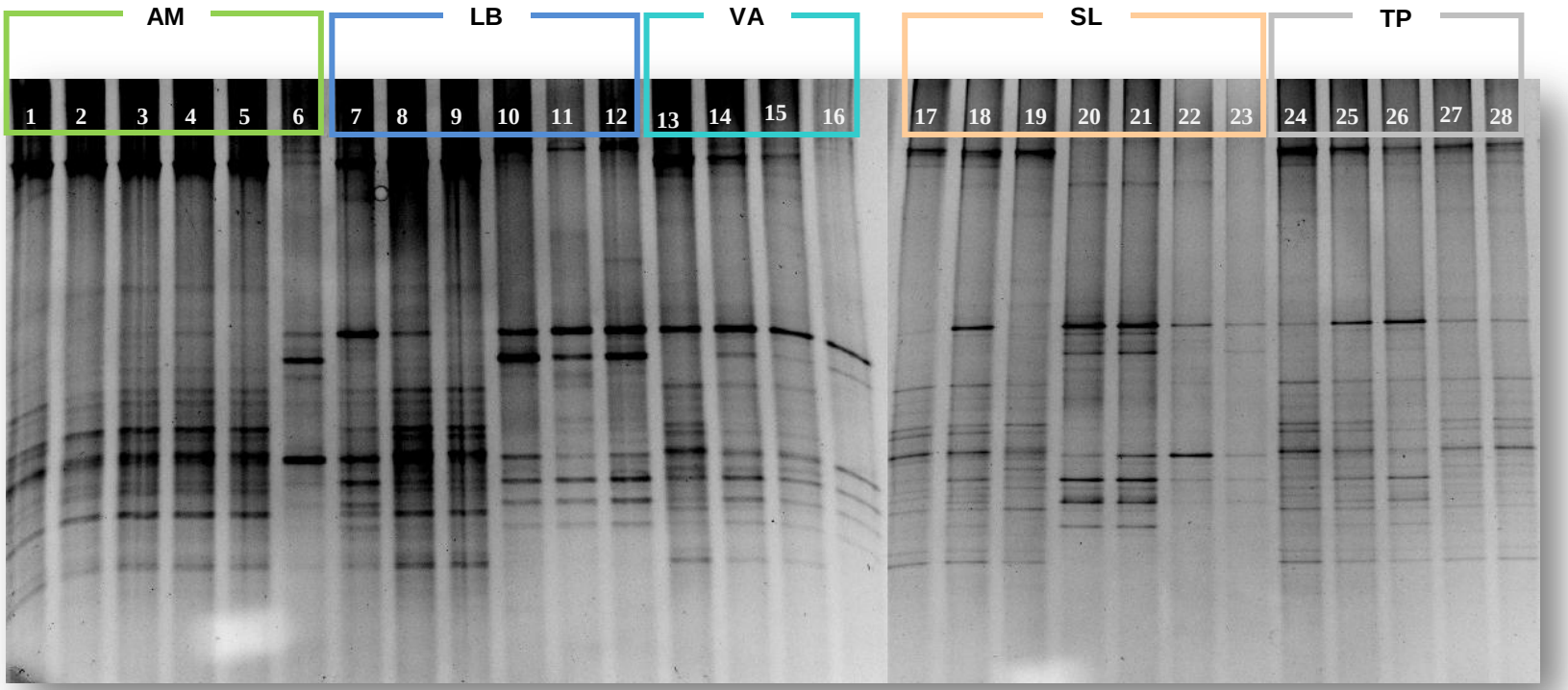

Figure 17- DGGE gel of PCR amplified 16S rRNA genes from skin samples from *P. perezi*.

The analysis of all the microbial community fingerprints showed that the five studied frogs populations do not group together based on their skin microbial community fingerprinting. Perhaps, DGGE profiling showed a characteristic community profile in frogs from the metal rich-effluent (Ribeiro da Agua Forte, Aljustrel) close to 80% of similarity. Other groups can be pointed, for example the AM samples cluster together except one near to 80% similarity.

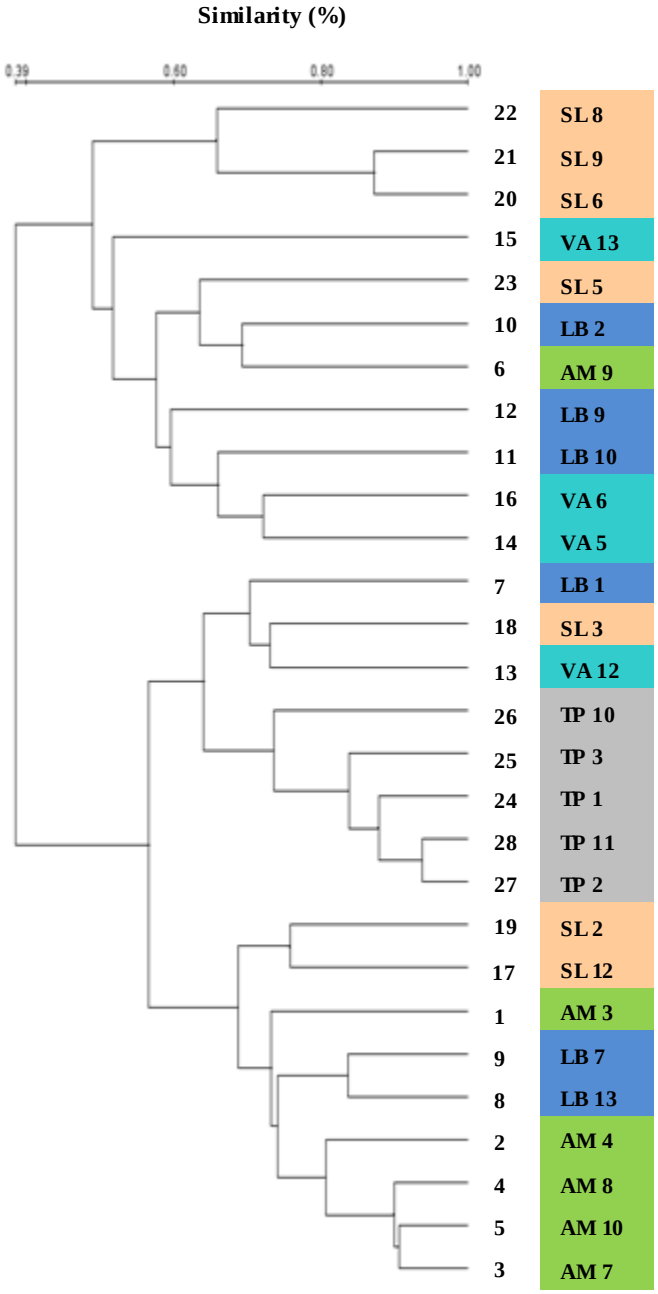

Figure 18- UPGMA dendrogram constructed using Dice's similarity coefficient generated from the DGGE profiles.

Cluster analysis was performed to gain an overview on the relatedness of phylogenetic profiles representing the communities of each frog population from each site (Figure 18).

This analysis corroborates the existence of a significant distinction between bacterial communities in frog skin from different populations. In fact, samples from the metal contaminated effluent clustered together. Samples from brackish water population (SL) seems not having a consistent relation with other sampled sites and samples from freshwater and good water quality tend to group together with high percentage of similarity.

3.5. Ecotoxicological assay

In total, 30 different bacterial isolates from LB (n=11), TP (n=10) and SL (n=9) were exposed to the metal-contaminated effluent. We also tested 15 isolates gram positive and 15 gram negative originated from the three different sampling sites.

On this ecotoxicological assay with bacteria it was used as criterion for metal resistence colony density and the growth rate .

3.5.1. Colony density

On this assay a bacterium isolate was considered resistant to the metal-contaminanted effluent when it grew after exposure to concentrations equal or above 50% of the effluent. Bacteria isolates were considered sensitive to metal contamination if their growth was inhibited in presence of metal contamination (effluent concentration higher than 50%).

The percentage of resistant isolates from the frog's microbial communities varieted among different populations and concentrations (Figure 19).

In a more generalistic view, 12 (40%) exposed bacteria did not exhibit growth in the presence of 25% of the metal contaminated effluent. As well, the majority of the isolates, about 18 (60%), did not grow when exposed to 100% of the contaminated effluent. On the other hand, bacteria belonging to the genus *Pseudomonas* (Sample Code: TP2_4) it was the only that growing until the minimum concentration of 25%. For the other resistant isolates tested, 18 of them grew at 50% and 12 can growths at 100% of effluent concentration.

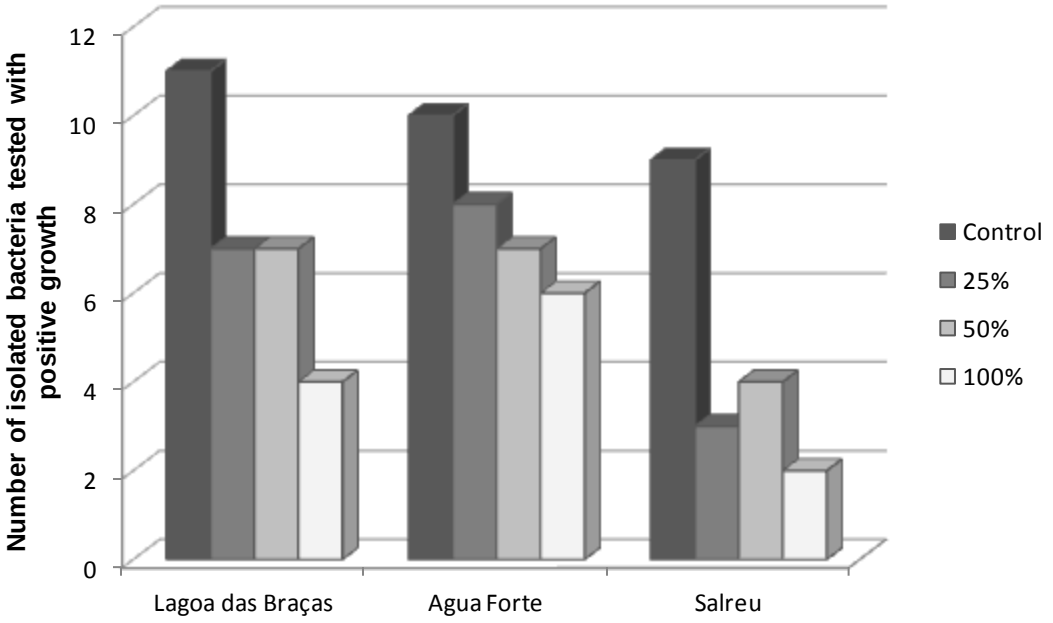

Figure 19- Number of Isolated bacteria exposed to three concentrations of contaminated effluent that demonstrated positive growth. (Lagoa das Braças (LB), Agua Forte (TP) and Salreu (SL)

When the resistance of bacterial isolates was evaluated at the population level the percentage of resistant isolates from the frog's microbial communities to contaminated effluent was higher in frogs from the contaminated site. About 60% of tested bacteria showed to be resistent to contamination levels present in effluent at 100%.

Bacterial isolates from SL population showed low tolerance to effluent exposure. Only 33% of bacteria from this site grew in the presence of 25% of effluent and 22% to an effluent concentration of 100%. Bacteria from LB seems to have an intermediate resistance comparing the three sampling sites.

Durind this ecotoxicological assay gram positive and gram negative bacteria were tested in a 1:1 porpotion. Results showed that in resistent bacteria to 50% effluent concentration, 13 of them present gram-positive staining and 4 gram-negative. For 100% of effluent, 9 were gram-positive and 3 gram-negative (Figure 20).

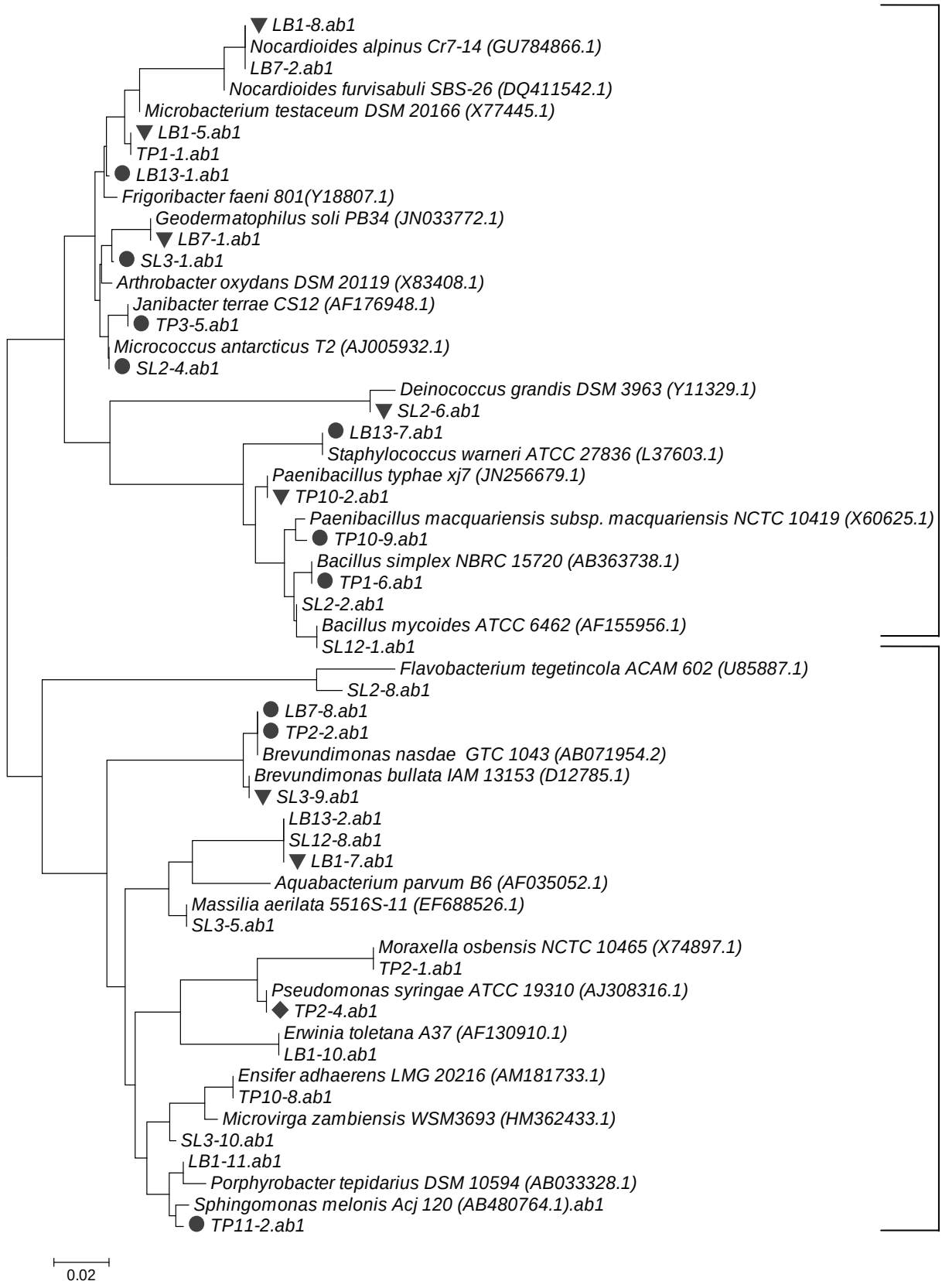

Figure 20- Genetic relation between bacterial isolates exposed to contaminated effluent. Different signs mark the growth at different concentrations. Bacterial isolate presents growth at: ♦- 25 % effluent concentration; ▼- 50 % effluent concentration; ● – 100 % effluent concentration.

3.5.2. Colony growth rate

Acording to the results obtained for growth rate, a significant inhibition was observed for oexposed to the metal-contaminated effluent. Compared with Control, bacterial suspension exposed to the effluent, exhibited a delay the appearance of colonies and the development was slower. Some bacteria showed this delay during all the 120 hours of exposure. Others, had a latency period and then they recovered to the same level of development of the corresponding control in all concentrations or excepting the highest. Also, for some bacteria isolates, latency period increased when exposed to the higher concentrations of effluent.

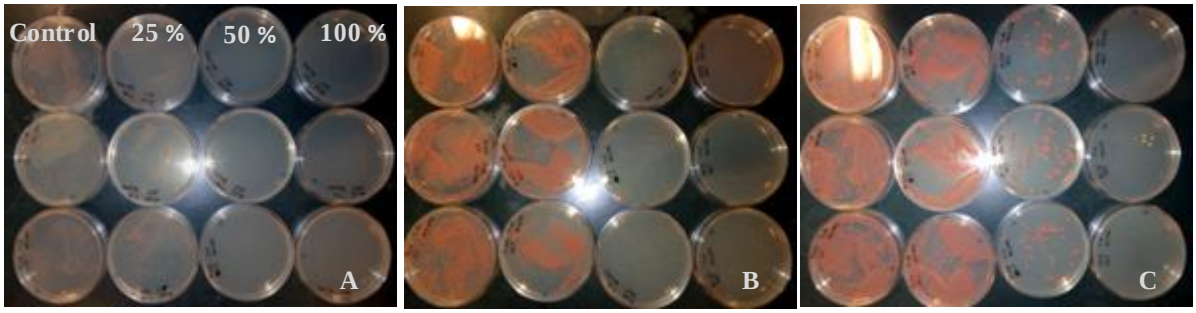

Figure 21- Images of the bacteria SL2_6 (*Deinococcus grandis,* 97.1% closest EzTaxon) isolated from the *Pelophylax perezi* skin during the toxicological assay with four different concentrations of metal-contaminated effluent. Image shows the growth of the bacteria after 1h of previous exposure to the effluent concentrations. (**A**) Growth after 48h. (**B**) Growth after 72h. (**C**) Growth after 120h (end of assay).

Facing the numbers obtained on point 3.5.1), the figures presented in Appendix A shows the different responces of bacterial isolates to effluent exposure. Graphs were performed using Microsoft Excel facilities and relates the growth categorization during the assay after exposure to the different concentration of metal contaminated effluent.

Table 10 - Results of ecotoxicological assays with contaminated effluent from site TP after 120 hours. Tolerance: R – Bacteria growths at 100% of effluent concentration; R$^-$ - Bacteria growths at 50% of effluent concentration. (-) – Growth absence; (=) - Growth rate approximately equal to control; (↓) – Growth rate decrease comparing to control.

Code	closest EzTaxon (Type strain)	%	Control	25%	50%	100%	Tolerance	Specific growth rate (μ) In the presence of the toxic	Gram-stained
LB1_5	*Microbacterium testaceum*	97.48	s+++	s+++	s+++	s+++	R	=	+
LB1_10	*Erwinia toletana*	98	s+++	-	-	-	-	-	-
LB7_8	*Brevundimonas nasdae*	99.83	s+++	s+++	s+++	s+++	R$^-$	-	-
LB1_7	*Aquabacterium parvum*	94.36	s+++	s+	s	-	R$^-$	-	-
LB7_2	*Nocardioides alpinus*	98.65	s+++	-	-	-	-	-	+
LB1_11	*Porphyrobacter tepidarius*	99.2	s	-	-	-	-	-	-
LB7_1	*Geodermatophilus obscurus*	95.09	s+++	s+	s+	-	R$^-$	↓	+
LB13_7	*Staphylococcus warneri*	100	s+++	s+++	s+++	s+++	R	↓	+
LB13_1	*Frigoribacter faeni*	99.01	s+++	s+++	s++	s++	R	↓	+
LB1_8	*Nocardioides furvisabuli*	98.66	s+	s	s+	-	R$^-$	↓	+
LB13_2	*Aquabacterium parvum*	94	s	-	-	-	-	-	-
TP1_1	*Microbacterium testaceum*	97.66	s+++	s+	s+	s+	R	=	+
TP1_6	*Bacillus simplex NBRC*	100	s+++	s+++	s+++	s+++	R	=	+
TP2_2	*Brevundimonas nasdae*	99.49	s+++	s+++	s+++	s	R	↓	-
TP11_2	*Sphingomonas melonis*	98.63	s+++	s+++	s+++	s	R	↓	-
TP2_4	*Pseudomonas syringae*	97.83	s+++	s	-	-	-	-	-
TP3_5	*Janibacter terrae*	98.48	s+++	s+	s+	s+	R	=	+
TP2_1	*Moraxella osloensis*	99.3	s(+++)	-	-	-	-	-	-
TP10_2	*Paenibacillus typhae*	98.07	s++	s+	s	-	R	↓	+

TP10_8	*Ensifer adhaerens*	97.24	s+++	-	-	-	-	-	-
TP10_9	*Paenibacillus macquariensis*	98.18	s+++	s+++	s+++	s+++	R	↓	+
SL2_6	*Deinococcus grandis*	97.18	s+++	s+++	s	-	R	↓	+
SL2_4	*Micrococcus antarcticus*	99.36	s++	s+++	s+++	s+	R	↓	+
SL12_8	*Aquabacterium parvum*	93.63	s+++	-	-	-			-
SL3_1	*Arthrobacter oxydans*	98.69	s+++	s+++	s+++	s++	R	↓	+
SL3_5	*Massilia aerilata*	99.1	s+	-	-	-	-	-	-
SL3_10	*Microvirga zambiensis*	96.5	s	-	-	-	-	-	-
SL2_2	*Bacillus mycoides*	98.9	s+++	-	-	-	-	-	+
SL2_8	*Flavobacterium tegetincola*	96.11	s+++	-	-	-	-	-	-
SL3_9	*Brevundimonas bullata*	99.68	s+++	-	s++	-	R	-	-

4. Discussion

4. Discussion

This study investigated the cutaneous microbial diversity of Perez' frog *Pelophylax perezi* with a focus on the bacterial microbiome resident on skin surface of individuals from five different populations distributed along several zones of the Portuguese territory. Population from the site Água Forte (TP) were exposed to a metal contaminated freshwater and populations inhabiting Salreu (SL) a water system with high salinity variations (brackish water) and low dissolved oxygen concentrations. It was already assessed in previous published works for this species, some effects that environmental stressors may cause directly to the host (Marques *et al.* 2008; Santos *et al.* 2013). However, indirect effects had never been addressed before for this specie or under this environment conditions

The structural microbial skin diversity of Perez' frog, *Pelophylax perezi* was assessed by cultivation in R2A medium and by denaturing gradient gel electrophoresis (DGGE) profiling, allowing the comparison between populations, under different methodologies. Identifying a common profile for individuals from the same population was expected. The fingerprint pattern of DGGE gels showed a high (>15) number of bands, illustrating a high number of bacterial species in frog's skin. Although, the dendrogram that was generated from culture-independent analysis based on DGGE community profiles did not clustered the cutaneous bacterial communities from the different individuals of the frog populations, except for AF population that cluster together and separately to the others. However, results showed groups of samples from similar water conditions, for example, AM samples tend to group with LB samples or LB with VA respectively with percentages of similarity between 60% and 80%.

Nevertheless, some bands were common to all profiles. All of the bacterial Phyla reported from previous studies on amphibian cutaneous bacteria where there were applied different methods, included *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Woodhams *et al.* 2007; McKenzie *et al.* 2011; Kueneman *et al.* 2013), which were also found in the present study. However, strains from *Deinococcus-Thermus* were only recovered in this study.

According to the results obtained from cultivation methods, the frog's skin microbial community exhibited a high percentage of bacteria belonging to *Proteobacteria*, *Actinobacteria,* and *Firmicutes* Phyla. By selecting morphologically different colonies after cultivation of solid medium, 38 different Genera, belonging to eight Classes, were identified in *P. perezi* skin. Analysing each site separately, only strains of the Genera *Brevundimonas* and *Sphingomonas* belonging to *Alphaproteobacteria* Class were

common to all the three frog sampled populations. The majority of bacterial isolates belonged to the Class *Actinobacteria* and *Alphaproteobacteria*. The frog population from Lagoa das Braças (LB) showed no isolates from *Deinococci*, *Flavobacteriia* and *Sphingobacteriia*. In the other hand, at the Genus level, TP population showed the lower diversity of bacteria isolated (n=15) and LB the highest (n=19).

Both SL and TP have different environmental characteristics when compared to the other sites studied. Salreu frog population, in a high salinity fluctuation environment presented most of the bacteria belonging to groups that are usually reported in salted environments. For example strains from the Genus *Paracoccus* (*Proteobacteria*), *Pseudorhodobacter* (*Proteobacteria*), *Flavobacterium* (*Bacteroidetes*), *Microvirga* (*Proteobacteria*) and *Deinococcus* (*Deinococcus-Thermus*) were found or isolated in high salinity locals as coastal seawater or Antarctic sea ice (Bowman *et al.* 1997; Khan *et al*. 2008; Uchino *et al*. 2002). Some groups were halophytic others were only tolerant to high salinity. *Deinococcus* are known as an extremophilic bacteria presenting high resistance to environmental stressors (Rainey *et al*. 1997).

Regarding to the microbiome isolated in TP frog population, some of the most common Genera that were found are known to include species able to be pathogenic to plants and animals, as for example: *Moraxella* (*Proteobacteria*), *Pseudomonas* (*Proteobacteria*) and *Staphylococcus* (*Firmicutes*) or linked to water and soil with poor quality and resistant to environmental contaminants as metals (Tan & Grewal 2001; Hedman *et al*.1993; Morris *et al.* 2008). The *Bacillus* (*Firmicutes*) genus was the most represented in this population and is characterized to include species able to cause disease to humans and animals (Suresh *et al.* 2004, Jensen *et al.* 2003).

In LB population, it is worth of notice the fact that several bacterial Genus appearing with high frequency, are related to those commonly found in freshwater systems (for example: *Aquabacterium*; Kalmbach *et al*. 1999 or *Nocardioides*; Zang *et al*. 2012). Overall, a few other phylotypes found on the amphibians also match sequences of bacterial strains that are known as plant symbiotic bacteria, typical from agricultural soil or water with high concentration of organic matter. Some of these include a strain of *Paenibacillus* (*Firmicutes*), *Cellulomonas* (*Actinobacteria*), and of *Azorhizobium* (*Proteobacteria*) (Kong *et al*.2013; Kang *et al*. 2007; Moreira *et al*. 2006)

Some questions are still not answered as the provenance and mode of acquisition of symbiotic bacteria living on the skin of amphibians. Several studies addressed the thematic, for example Walke *et al.* (2011), found that there were similarities between the microbial structure of the skin in adults and egg masses of *H. collymbiphyllum,* suggesting vertical transmission within the species concerned. However, other ways of

microbioma acquisition through environmental via was not discarded. Lauer *et al.* (2007) showed that bacteria rinsed from the *P. cinereus* skin were different from bacteria that efectively colonized the skin and Banning *et al*. (2008) demonstrated that may not exist a linear transposition between the microbial profile found in the surrounding environment and amphibian skin community. It is evident that the majority of phylotypes found on amphibians are similar to typical bacterial strains of the soil, plant symbiotic bacteria and water body systems. Therefore, it is expected that there is a set of factors shaping the frog microbial structure.

In this study we found that there were differences in various aspects in the microbial community associated to the skin of *P. perezi*. McKenzie *et al.* (2011) investigated three species of amphibians from different ponds hoping that frog species that co-inhabit the same habitat, thus in the presence of the same bacterial pool, shared the same structure of cutaneous microbiome. What these authors discovered was that there was specificity between the symbiotic bacteria and host species as well as individuals of the same species had the same microbiome from population to population and also reported that this specificity differed between species.

Various authors addressed the theme of the relationship between the amount of bacteria that produce antimicrobial substances and resistance of frog individuals against several pathogens. Studies of bacteria removal from *Plethodon cinereus* skin demonstrated that individuals with reduce microbiota had a loss of body mass and higher morbidity than individuals with unmanipulated microbiota when exposed to the fungus *Batrachochytridium dendrobatidis* (*Bd*) (Becker & Harris 2010). As well, the bioaugmentation of the skin of mountain yellow-legged frog, *Rana muscosa* with anti-*Bd* bacteria prevented morbidity and mortality comparing with those not treated with anti-*Bd* bacteria (Harris *et al*. 2009).

In fact, these results demonstrated that not only bacteria diversity present in amphibian skin can provide a better protection against emerging infectious pathogen *B. dendrobatidis* (*Bd*) or other pathogens but also the quantity/density of antifungal cutaneous microbes could be determinant. In the present work, growth inhibition assays to measure antimicrobial activity of cutaneous bacteria was not described but should be assessed in future works aiming a more complete and accurate understanding of the functions of the frogs microbiome.

Comparing frog populations, results showed differences in the density of heterotrophic cultivable bacteria. On average, individuals from SL population registered the highest density of CFU per sampled frog and TP the lowest density. In the same way, on

average, adult individuals of *P. perezi* from SL harboured approximately nine morphological distinct types of bacterial colonies (N=7, range 7-11, SD 0.63) and TP showed a mean of five morphological types of colonies (N=5, range 3-6, SD 0.58).

Analysing these results with culture independent methods can evidence that bacterial communities are effectively different. Regarding to the metal contaminated site, results suggest that environmental contamination can modify the structure of skin bacterial communities. For example, DGGE profiles related to TP population presented the highest number of bands. On the other hand, when analysing the culturable portion of the skin bacterial community at TP population it presented the lower number of CFU and low colonial diversity. It can suggest that in fact, contamination cannot decrease the quantity and diversity of bacterial community but modify the microbial structure and composition of frog's skin microbiome. Perhaps, more investigation should be done to accurate these results.

Blaustein *et al*. (2003; 2010) reviewed how environmental change and environmental stressors contribute, direct or indirectly, to the global amphibian decline. Countless factors contribute to increase disease susceptibility and this work suggests other effects of environmental contamination that may contribute for the increase amphibian vulnerability, disrupting their primary defence barrier.

Furthermore, the objective of the present work was to assess the cutaneous microbial diversity, compare frog populations and verify if there was a significant difference in this community when frog population was exposed to environmental stressors as a metal rich effluent. As expected, chemical contamination exhibited negative effects in the skin microbiome of *P. perezi* suggesting that populations exposed to this sort of environmental severe disruption have less protection than the another populations without environmental contamination.

In a second stage of this work, some of the bacterial strains isolated (recovered) during population community characterization were exposed to a mining effluent, Àgua Forte stream near the Aljustrel tailing. Physico-chemical determinations on water samples from the same pond revealed low pH (4.93), high conductivity (2210 µS.cm $^{-1}$) and high metal concentrations (Al, As, Cd, Cu, Cr, Fe, Ni, Pb and Zn). Bacterial toxicity assay showed different responses of bacterial strains isolated from skin of frogs belonging to the three populations studied (Fig. 19). Tested bacteria isolated from TP population (60%) showed to be more resistant to exposure at acidic TP effluent without dilution. Bacteria from SL population demonstrated low tolerance to effluent (22% growth at 100% effluent) and LB had an intermediate response (36%).

As well, comparing bacterial growth between control and exposed bacteria, we could notice a delay in the appearance of colonies and development was slower. Some bacteria showed this delay during the entire assay. Others exhibited a higher resilience and after a latency period they recovered to the same level of development of the corresponding control in all concentrations or with the exception of the highest. In general, gram positive bacteria were less sensitive to the effluent. Consistent with data, bacteria isolated from the frog population with salinity fluctuation had lower tolerance to contaminated effluent than those of the other freshwater population. This suggests a selected colonization of the frog skin accordingly to habitat chemical conditions. Although host species skin characteristics and developmental stage are considered were the major factor affecting the amphibian skin microbiota (Kueneman *et al.* in press), the environment, as a whole, also exerted a statistically significant effect. Cutaneous bacteria of *P. perezi* differed significantly between populations whose habitat presented environmental stressors. Salinity, low pH, high conductivity or high concentration of metals can act as environmental shapers of the diversity and structure of skin microbial community. It also should be considered that amphibian skin is the substrate to resident bacteria and alterations in the environment can also cause stress in the host, alteration of skin secretions and skin integrity (Pessier 2002) and therefore the alteration of optimal conditions to symbiotic bacteria.

Abiotic alterations, in environment, cause alterations in bacterial community structure, diversity and dynamics of water or soil. This work provides information that allow us to assume that this kind of alterations also influences symbiotic cutaneous bacteria present in *P. perezi* frog. In addition, sublethal stressors as xenobiotic (e.g. metals) or acidification may compromise amphibian immune system and increase susceptibility to disease (Carey *et al*. 1999), under the same perspective, stressors and pathogenic agents act synergistically.

In spite of the existing limitations, DGGE is still an adequate approach to compare microbial communities. Cultivable-dependent methods give complementary data information, but only about the cultivable part of the microbial bacterial community. Because of isolation procedure, with only one type of culture medium, and because not all the bacteria could be cultured, we cannot represent here the entire resident bacterial community present in the frog skin. Additionally, it is possible that not all of the swabbed bacteria are actual skin symbionts of the amphibians. Nevertheless, since other works showed that amphibian species inhabiting the same pond harboured distinct bacterial communities, a significant proportion of the sequences were host-associated and not simply transient microbes from the pond environment.

Ecotoxicological assays with bacteria isolated from frog skin is a new methodology in this kind of ongoing studies, but a necessary approach to assessed if frog outer (skin) bacteria can resist face to a contamination.

This kind of approach is important to understand the mechanisms that drive the diversity of cutaneous bacteria in different species and populations, and the environmental factors that cause modification on these communities. A more accurate experiment should be done to know how cutaneous bacteria are affected and more population under stress should be assessed to known if different contaminant has different effects. In the same way, in a scenario of growing environmental pollution and stress, these evaluations are important to predict which species or population is more vulnerable. The importance of antimicrobial peptides and symbiotic bacteria is already known from previous works, but it is the iceberg tip of an extensive exploratory work. Bacteria found in populations studied in this work showed a majority of colorfully bacteria. Bacterial pigments play an important role of cell protection, e.g. absorb UV radiation or quench oxygen free radical. Also confer antibacterial and metal resistance. In relation to this, can amphibian somehow benefit from this sort of protection too?

Conclusion

Conclusion

The present work showed variability among populations in the microbial community of *Pelophylax perezi*. Also, it was observed that some within population variability may exist, though not as high as that of among population. Furthermore, it was observed that frog populations exposed to environmental stressors, like increased salinity or metal contamination has a microbial community particularly correlated with those environmental parameters. This evidence suggests a strong influence of environmental factors in the modulation of cutaneous bacterial community and also that it plays an important role in the potential selection of bacteria that effectively colonizes the skin of this amphibian species.

In addition, this work contributes to understand that environmental chemical contamination, like metals contamination, may cause negative effects on skin resident bacteria. Therefore, it is suggested that such effects can contribute for destabilization of bacterial community harmony and jeopardize the protection effect of this skin barrier in amphibians, making them more susceptible to opportunistic disease. This is a possible explanation for why some amphibian populations are more resistant to disease than others, and, thus, synergetic effects should be considered when assessing ecological risks in amphibians.

More investigation is needed to understand these processes: other amphibian species should be studied; other pollutants or environmental stressors have to be investigated and other assays should be done, for example evaluate the cutaneous bacterial community before and after the exposure to several potential environmental pollutants.

Appendix A

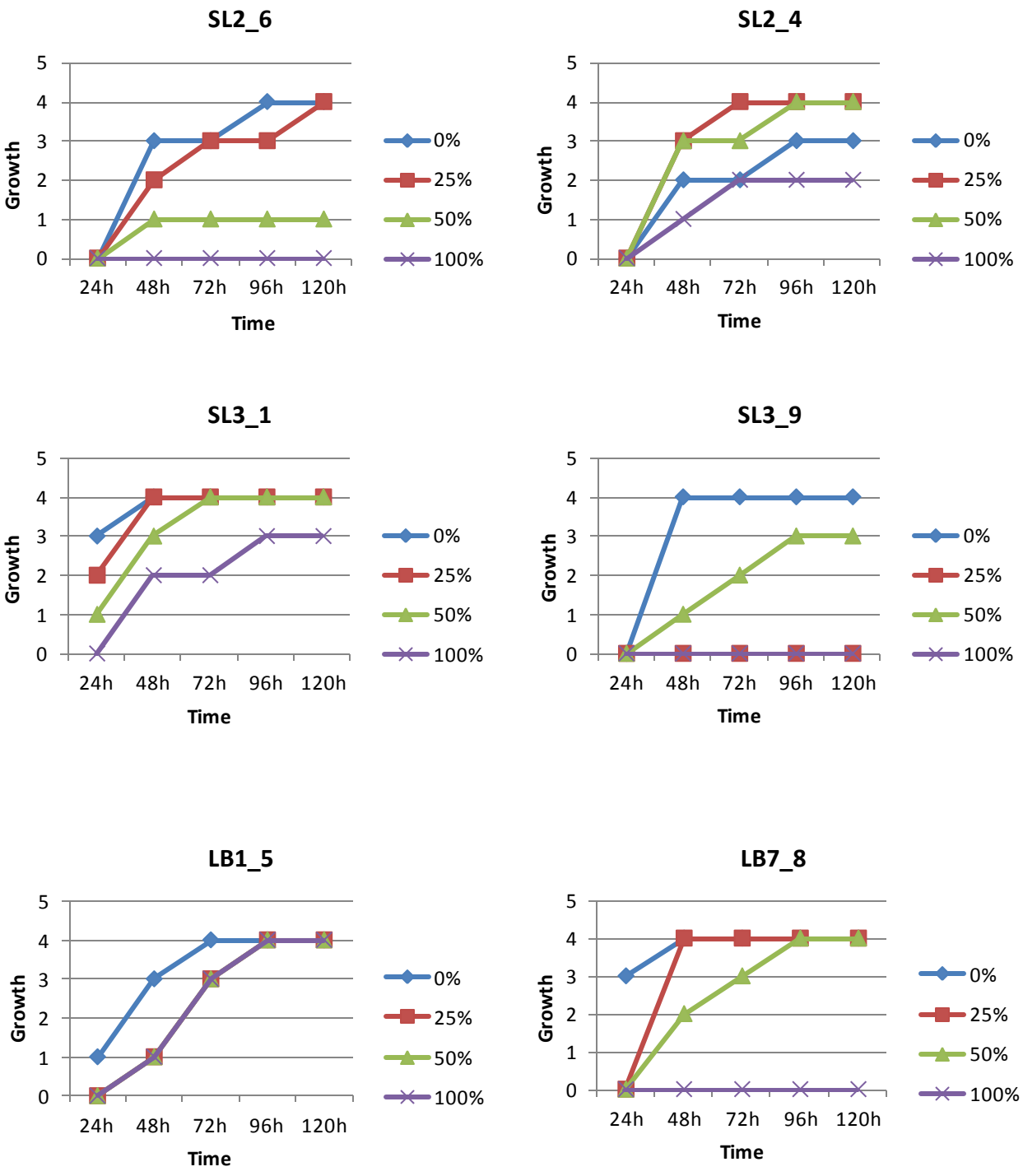

Figure A1 – Graphs representing the growth of tested isolated bacteria during ecotoxicological assays. This graphs correspond to bacteria from different sampled populations (SL= Salreu; LB = Lagoa das Braças and AF= Agua Forte). Also correspond to bacterial isolates considered resistant, once they present growth in 50% and 100% concentration of contaminated effluent (continue next page).

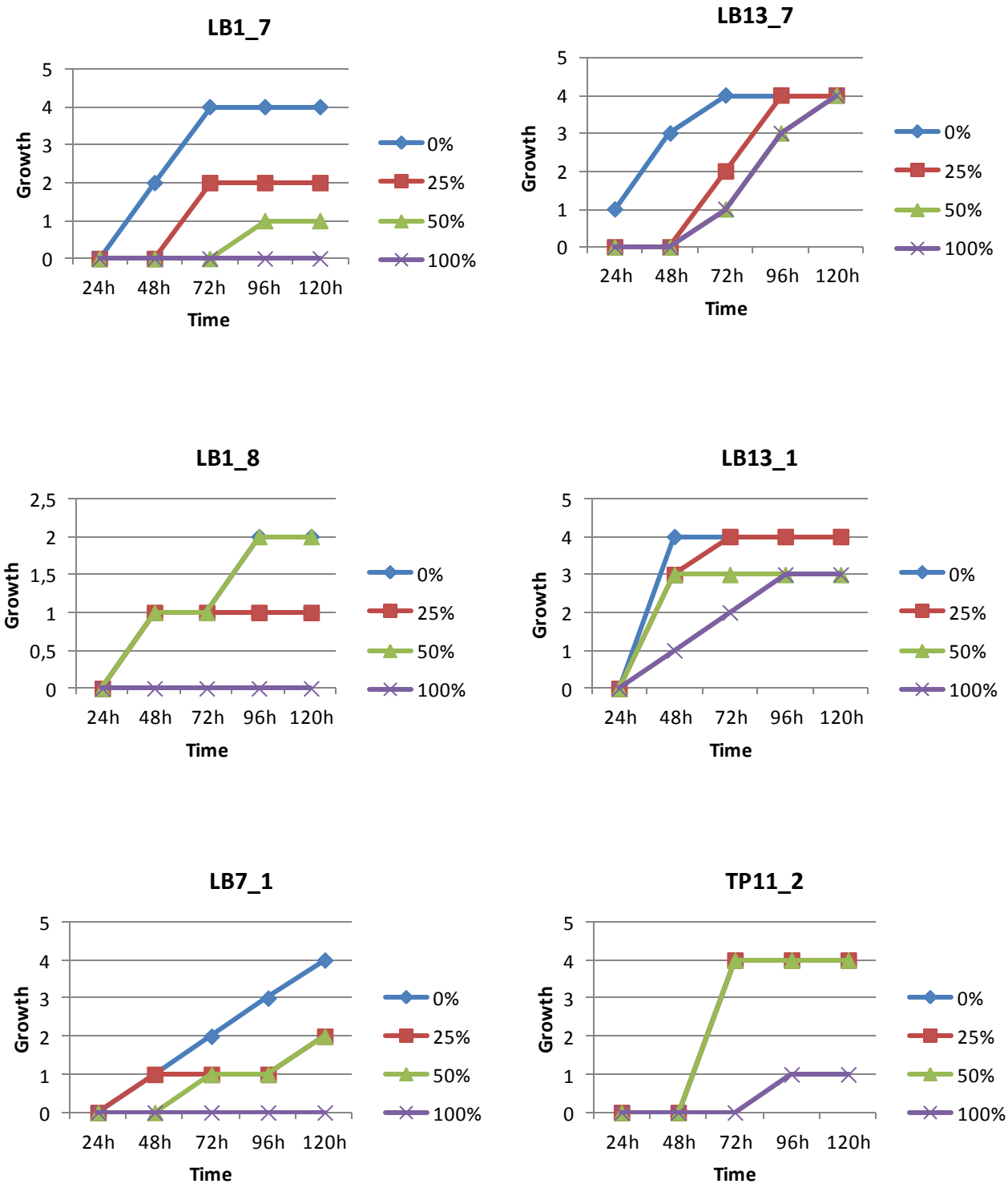

Figure A2 - (Continuation) Graphs representing the growth of tested isolated bacteria during ecotoxicological assays. This graphs correspond to bacteria from different sampled populations (SL= Salreu; LB = Lagoa das Braças and TP= Agua Forte). Also correspond to bacterial isolates considered resistant, once they present growth in 50% and 100% concentration of contaminated effluent (continue next page).

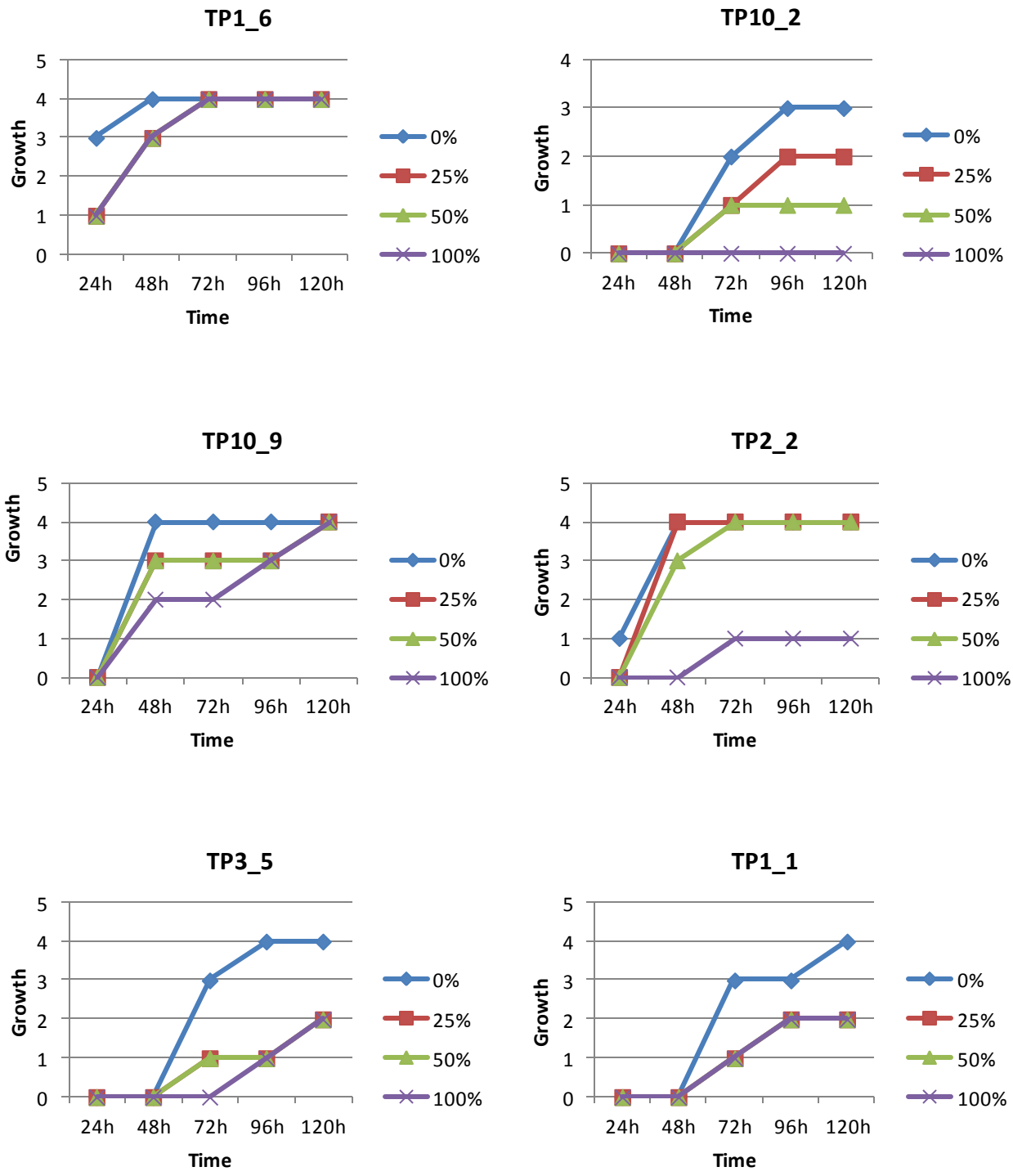

Figure A3 - (Continuation) Graphs representing the growth of tested isolated bacteria during ecotoxicological assays. This graphs correspond to bacteria from different sampled populations (SL= Salreu; LB = Lagoa das Braças and TP= Agua Forte). Also correspond to bacterial isolates considered resistant, once they present growth in 50% and 100% concentration of contaminated effluent.

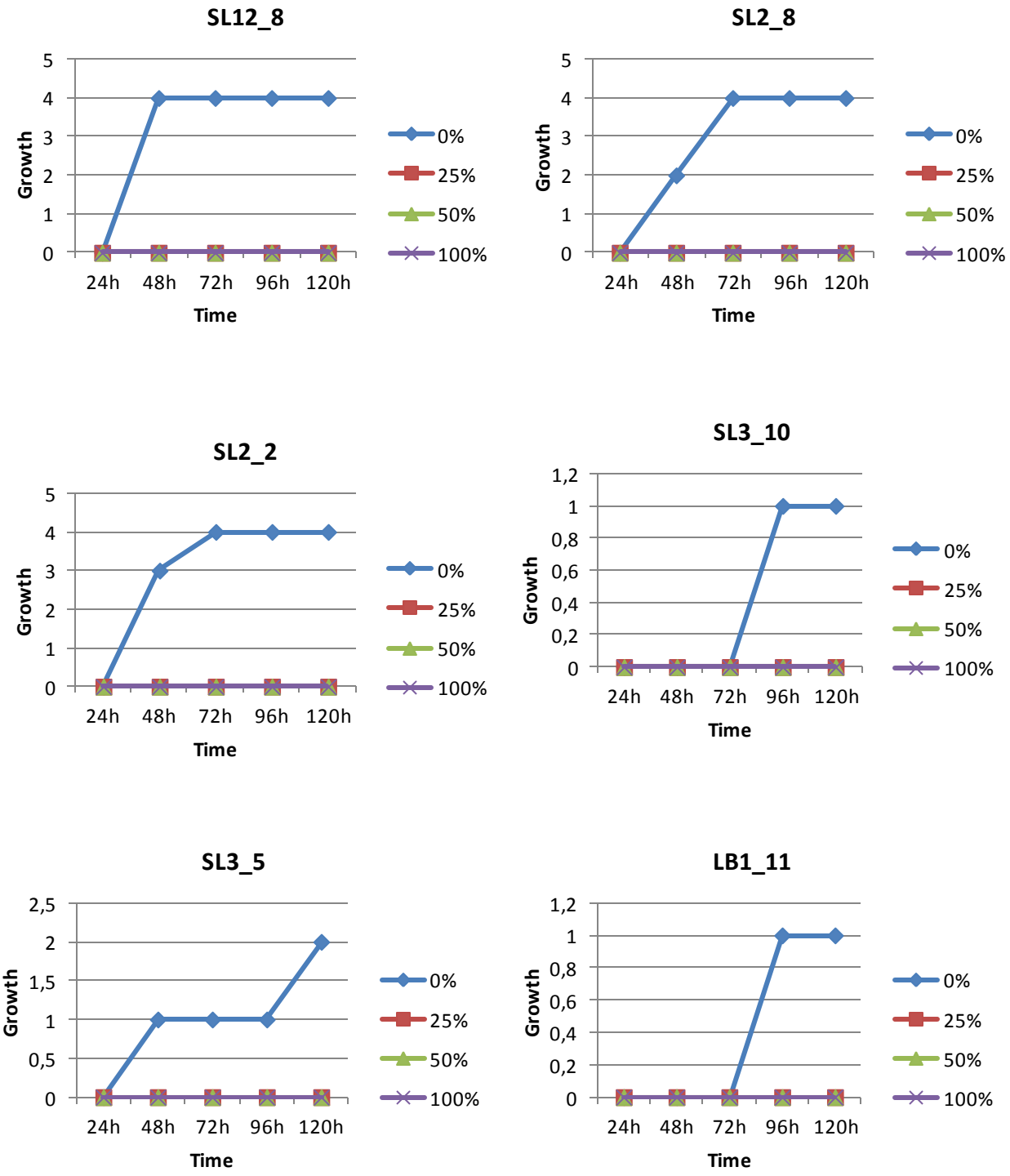

Figure A4 - Graphs representing the growth of tested isolated bacteria during ecotoxicological assays. This graphs correspond to bacteria from different sampled populations (SL= Salreu; LB = Lagoa das Braças and TP= Agua Forte). Also correspond to bacterial isolates considered non resistant, once they present growth in 0% (Control) and 25% concentration of contaminated effluent (continue next page)

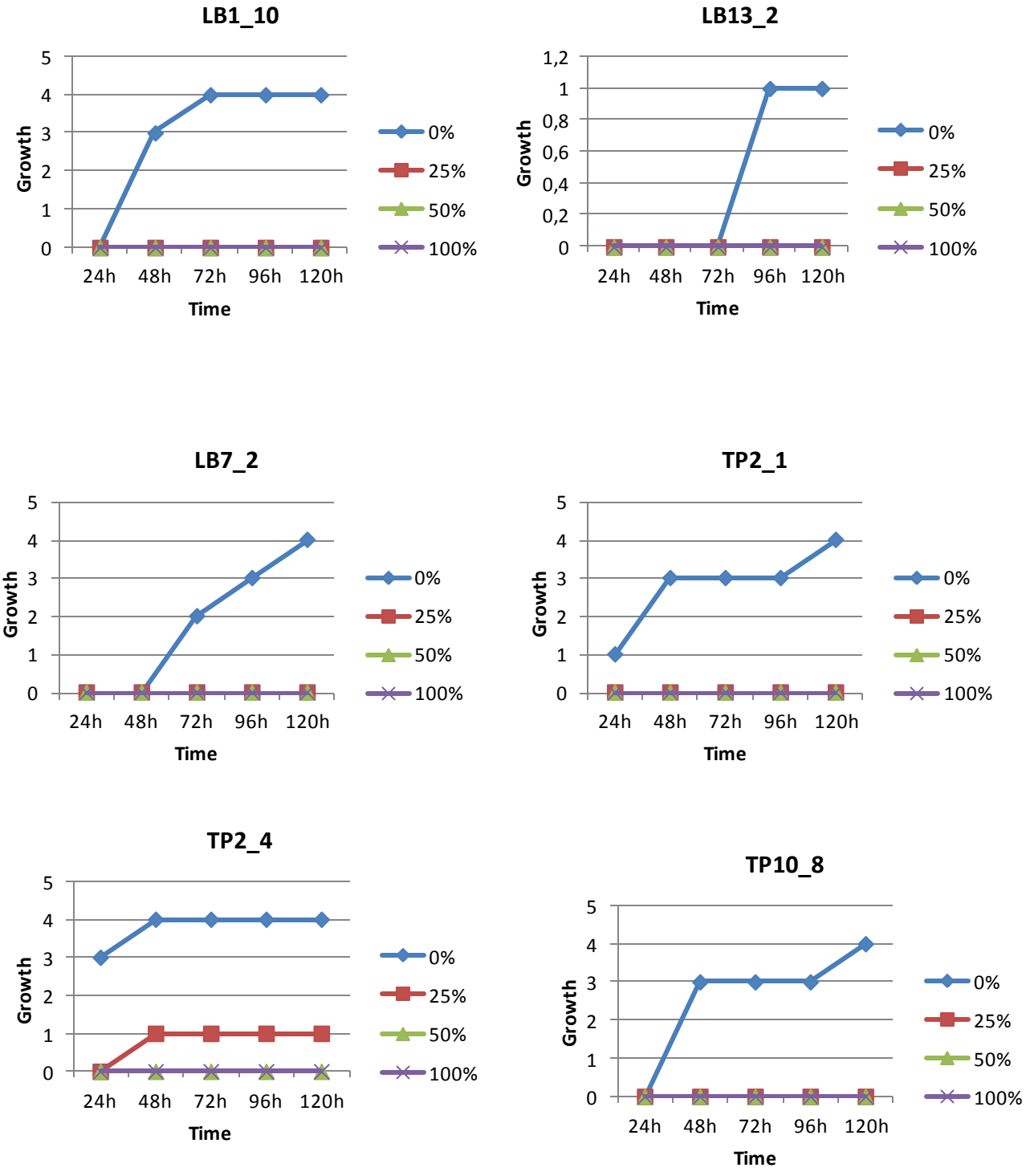

Figure A5 - (Continuation) Graphs representing the growth of tested isolated bacteria during ecotoxicological assays. This graphs correspond to bacteria from different sampled populations (SL= Salreu; LB = Lagoa das Braças and TP= Agua Forte). Also correspond to bacterial isolates considered non resistant, once they present growth in 0% (Control) and 25% concentration of contaminated effluent.

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